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DYE-SENSITIZED PHOTOINACTIVATION OF ACETYLCHOLINESTERASE

BY

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MAXWELL D. CUMMINGS

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

The photoinactivation of acetylcholinesterase was sensitized by various xanthene dyes. Photosensitizing effectiveness paralleled the triplet state quantum yields of the dyes studied. Various mechanistic tests including the use of azide, deuterated buffers and argon gassing to remove oxygen were employed in an attempt to distinguish between Type I (radical) and Type II (singlet oxygen-mediated) reaction pathways. The Type II pathway was found to predominate under aerobic conditions whereas an inherently faster Type I mechanism facilitated photoinactivation under anaerobic conditions. Dye-enzyme complex formation was demonstrated by absorption spectroscopy and fluorescence techniques under aerobic conditions. Dissociation constants calculated from both the kinetic and spectroscopic data were of comparable magnitude.

Protection from photoinactivation by the competitive inhibitor edrophonium, the pH dependence of the photoinactivation rates as well as the results of various amino acid analyses indicated that a crucial histidine residue was involved in the photoinactivation process. Tryptophan destruction under aerobic conditions was unequivocally demonstrated by both spectroscopic methods and amino acid analyses and by amino acid analyses (only) under anaerobic conditions. Tyrosine was not involved in the photoinactivation process under any of the conditions studied. Methionine may be involved in the apparently less specific photoinactivation mechanism observed at lower pH values.

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TABLE OF CONTENTS

Page

CERTIFICATE OF EXAMINATION ii

ABSTRACT iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

ABBREVIATIONS vii

INTRODUCTION 1

i) History 1

ii) Photosensitized Oxidations: Reaction Pathways and Photosensitizers 3

iii) Photosensitized Oxidations: Distinguishing Type I and Type II Pathways 6

iv) Photosensitized Oxidations: Susceptible Amino Acids 10

v) Photosensitized Oxidations: Protein Studies 12

vi) Photosensitizers: Psychological and Neurological Effects . . 16

vii) Summary 19

MATERIALS 20

METHODS 24

i) Purification of *E. electricus* AChE 24

ii) Routine Assays for AChE Activity 24

iii) Active Site Determination 25

iv) Electrophoresis 25

v) Densitometry 26

vi) Photoinactivation Studies 27

Page

vii) Dye-binding Studies 29

viii) Calculation of Apparent Dye-AChE Dissociation Constants . . 30

ix) Amino Acid Analyses 31

RESULTS & DISCUSSION 32

i) Purification of AChE 32

ii) Sensitized Photoinactivation: Xanthenes 35

iii) Sensitized Photoinactivation: Erythrosin 35

iv) Erythrosin-sensitized Photoinactivation: Azide, Argon and D_2O 39

v) Spectroscopic Studies 44

vi) Protection From Photoinactivation by Edrophonium 49

vii) The pH Dependence of Photoinactivation 49

viii) Amino Acid Analyses 52

CONCLUSION 58

REFERENCES 60

ABBREVIATIONS

AChE	Acetylcholinesterase (E.C.3.1.1.7)
ASCh	Acetylthiocholine
DTNB	5,5'-Dithiobis-(-2-nitrobenzoic acid)
D_2O	Deuterium oxide
DCl	Deuterium chloride
NaOD	Sodium deuteroxide
D_3PO_4	Phosphoric acid-d_3
DTT	Dithiothreitol
DMB	Decamethonium bromide
EQB	Equilibration buffer
M7C	N-Methyl-7-(dimethylcarbamoxy) quinolinium iodide
M7H	N-Methyl-7-hydroxyquinoline
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

History

In 1888 Marcacci (1) presented the first documented report of damage to and destruction of a variety of biological processes in the presence of light, a chemical sensitizer and oxygen. He observed that various alkaloids produced light-dependent damage to amphibian eggs, the development of grain and several other systems. Following this line of investigation Raab, in 1900 (2), showed that dyes such as eosin and acridine, which had no effect on paramecia in the dark, rapidly killed these microorganisms in the light.

The first studies on protein preparations were carried out by von Tappeiner in 1903 (3) and von Tappeiner and Jodlbauer in 1904 (4). The results they obtained for the erythrosin-sensitized photoinactivation of the enzyme invertin as well as for the sensitized 'photokilling' of bacteria (5) demonstrated conclusively that these processes were oxygen-dependent. These reports came at a time when there was much interest in photobiological processes and served to stimulate research in this field (6). Some of this interest stemmed from the hypothesis that the mechanisms of photosensitization resembled those of naturally occurring physiological photobiological processes but no evidence has been acquired since that time to support such a supposition (6).

Mechanistic studies of sensitized photooxidations were initiated by several investigators in 1926 (12). Between 1931 and 1935 Kautsky published the results of studies involving discretely immobilized sensitizers and substrates (10). Kautsky proposed that singlet oxygen

was the intermediate in these reactions but this idea was not widely accepted until 1964 when it was conclusively established by the work of Foote and Wexler (10).

In 1951 Weil *et al.* (7) published the results of a comprehensive study of the light- and oxygen-dependent effects of the thiazine dye methylene blue on various amino acids. Photooxidations were monitored by oxygen consumption and ammonia liberation and the effects of pH, methylene blue concentration and methylation of the α-amino groups of the amino acids were studied. These investigators found histidine, methionine, tryptophan, tyrosine and cystine (under the reaction conditions employed cysteine was rapidly oxidized to cystine by atmospheric oxygen) to be susceptible to methylene blue-sensitized photooxidation whereas the other amino acids reacted very slowly or not at all, depending on the reaction conditions. One exception to this generalization was the observation that methylation of the α-amino group of lysine greatly increased the susceptibility of this otherwise unreactive amino acid to sensitized photooxidation. Studies on glycine oligopeptides indicated that peptide bonds were not cleaved in these sensitized photooxidations under any of the reaction conditions employed (7). The findings and interpretations of these investigators are still relevant today.

In a 1972 review, Westhead (8) stated that the techniques introduced by Weil *et al.* in 1951 were seen by some as having great potential for use in active-site characterizations. Xanthenes, thiazoles, thiazines, acridines, porphyrins and riboflavin had been reported to act as photosensitizers in 1959 (9) or earlier (e.g. 1-7) and the procedures involved were relatively simple. However, this

potential was never developed to any great degree due mainly to the lack of a standard system for these types of studies, which would have facilitated the comparison of experimental results. Furthermore, the 1960's saw the development of labelling reagents with greater specificity than that typically observed with the commonly employed photosensitizers. Westhead (8) and other reviewers (e.g. 10) have also noted the large number of incomplete mechanistic studies of the photosensitized oxidation of proteins in contrast to the relatively few cases in which mechanisms and susceptible sites or residues have been conclusively established. It seems clear, however, that under the proper experimental conditions, the finding of which in itself may be a time-consuming task, dye-sensitized photooxidations can be used to obtain useful knowledge about proteins as well as the mechanisms involved in photosensitization.

Photosensitized Oxidations: Reaction Pathways and Photosensitizers

Photosensitized oxidations are generally described in terms of the two pathways shown in Figure 1. As the diagram implies, the two pathways may be competitive; this often depends on the system being studied and/or the reaction conditions (10).

The Type I pathway involves the direct interaction of the triplet excited state of the sensitizer with an oxidizable substrate such as an amino acid or protein thus leading to radical formation. In this pathway molecular oxygen reacts with the substrate radical whereas the Type II pathway involves quenching of the triplet excited state of the

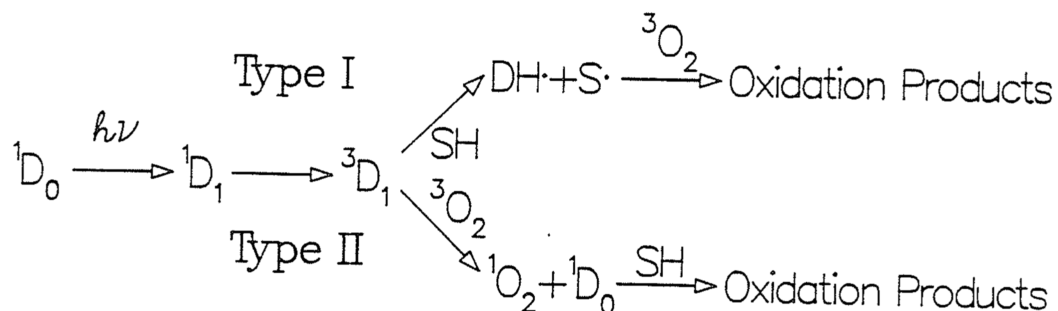


Figure 1. Photosensitized oxidations: Type I and Type II pathways. $^1D_0$ = ground state sensitizer, $^1D_1$ = first excited singlet state of sensitizer, $^3D_1$ = first excited triplet state of sensitizer, SH = oxidizable substrate, $^3O_2$ = ground state oxygen, $^1O_2$ = first excited singlet state of oxygen, DH· = sensitizer radical, S· = substrate radical.

sensitizer by ground state molecular oxygen thereby generating singlet oxygen . This excited oxygen species then reacts with an oxidizable substrate (6,10,11). The first excited state of oxygen ($^1\Delta_g O_2$) is believed to be the only important form of singlet oxygen in photosensitized oxidations (12). The implication of the first excited state of oxygen ($^1\Delta_g O_2$; 22.6 kcal mol^{-1}) as the oxygen species involved in Type II photosensitizations derives from the fact that dyes with triplets of lower energy than that of the second excited state of oxygen ($^1\Sigma_g O_2$; 37.7 kcal mol^{-1}) have been reported to sensitize photooxidations *via* Type II mechanisms (12). In addition, the rapid decay of $^1\Sigma_g O_2$ in aqueous solutions makes its involvement in photosensitized oxidations unlikely (10). In the remainder of this thesis the term singlet oxygen will refer to the first excited state of oxygen.

Straight and Spikes (10) as well as others (e.g. 13) have described a variety of alternative and often competing reaction routes which together comprise the Type I reaction pathway. Although these various reaction mechanisms may increase the complexity of both the overall reaction mechanism and the products obtained, the studies herein described were aimed at distinguishing between the Type I and Type II pathways shown in Figure 1. To avoid unnecessary complexity this discussion will be confined to the minimalistic scheme shown in Figure 1.

Photosensitizers of biomolecules include both natural and synthetic compounds such as petroleum products, aromatic hydrocarbons, furanocoumarins, thiophenes, organo-metallic complexes, inorganic ions as well as the commonly studied halogenated xanthenes and the thiazine methylene blue (10,15,16). Good photosensitizers can be generally

described as molecules which have a high triplet state quantum yield (Φ_T). Figure 1 shows that the triplet excited state of the photosensitizer is the second and final intermediate in that part of the photosensitization process which is common to both the Type I and Type II pathways. The structures of several xanthene dyes and some relevant photochemical parameters are shown in Figure 2 and Table 1 (adapted from 14). This family of dyes shows increasing quantum yields of both the triplet excited state and singlet oxygen due to increases in both the number and atomic weight of the halogen substituents. These trends can be ascribed to an internal heavy atom effect (17), whereby the addition of halogens to the xanthene moiety serves to enhance the rates of spin-forbidden processes such as the conversion of the first excited singlet state of the dye to its first excited triplet state (see Figure 1).

Photosensitized Oxidations: Distinguishing Type I and Type II Pathways

Many of the studies on photosensitization of biomolecules have focussed on the determination of the reaction pathway involved (Type I or Type II) and/or the extent to which each of these pathways contributes to the overall process. Two commonly used diagnostics for distinguishing between the two pathways are based on the efficient quenching of singlet oxygen by the azide ion and the enhanced lifetime of singlet oxygen in deuterium oxide (D_2O). It must be stressed that a single test under one set of reaction conditions will not be sufficient to conclusively differentiate between a Type I and Type II mechanism -

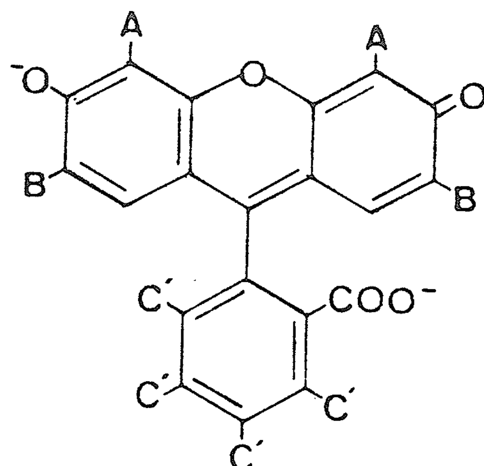


Figure 2*. General structure of xanthenes showing common sites of halogenation (A,B,C - see Table 1).

Common Name	A	B	C	Φ_T(absolute)[1]	$\Phi_{^1\Delta_g O_2}$(relative)[2]	λ_{max}(nm)
Rose bengal	I	I	Cl	0.86	1.0	548
Erythrosin	I	I	H	0.69	0.89	526
Eosin Y	Br	Br	H	0.37	0.79	516
Eosin B	Br	NO_2	H	0.32	0.69	517
Fluorescein	H	H	H	0.03	0.04	490

Table 1*. Halogen substituents and some physical parameters of several common xanthene photosensitizers.

[1] Quantum yield of dye triplet state formation.

[2] Relative quantum yield of singlet oxygen production.

*Adapted from (14).

several different tests under varied conditions are recommended (10).

Although it seems to be agreed that the lifetime of singlet oxygen is ten times greater in D_2O than in H_2O the exact values of these lifetimes seem to be controversial (compare 18-20 with 21). Regardless of the exact values it is commonly assumed that a ten-fold rate increase can be expected for a Type II photosensitization when the solvent is changed from H_2O-based to D_2O-based. This expectation assumes that the lifetime of singlet oxygen is rate-limiting in both systems (10) and also that all other pertinent factors will be identical in the two solvent systems. These assumptions may not always be valid and it seems possible that other 'D_2O-effects' could be present. Nevertheless this enhancement of reaction rates in D_2O-based systems has been very useful in the elucidation of the mechanisms of photosensitized oxidations.

The quenching of singlet oxygen by the azide ion is such that in a 10 mM azide solution the lifetime of singlet oxygen is reduced to between one-fifth (21) and one-tenth (20) of its natural lifetime in an H_2O-based solvent system with no azide present. Azide ion is commonly employed as a singlet oxygen scavenger in mechanistic studies wherein an azide-dependent rate reduction is interpreted as being indicative of the involvement of singlet oxygen as an intermediate in the reaction. The partial protection of the oxidizable substrate has been interpreted as an indication of a mixed Type I-Type II mechanism (11,22-24).

Another seemingly obvious but apparently uncommon approach to distinguishing between a Type I and Type II reaction pathway or to determining the relative significance of each pathway in a photosensitized oxidation is to study the effects of oxygen removal. Saturation of the reaction medium with a photochemically unreactive gas and the

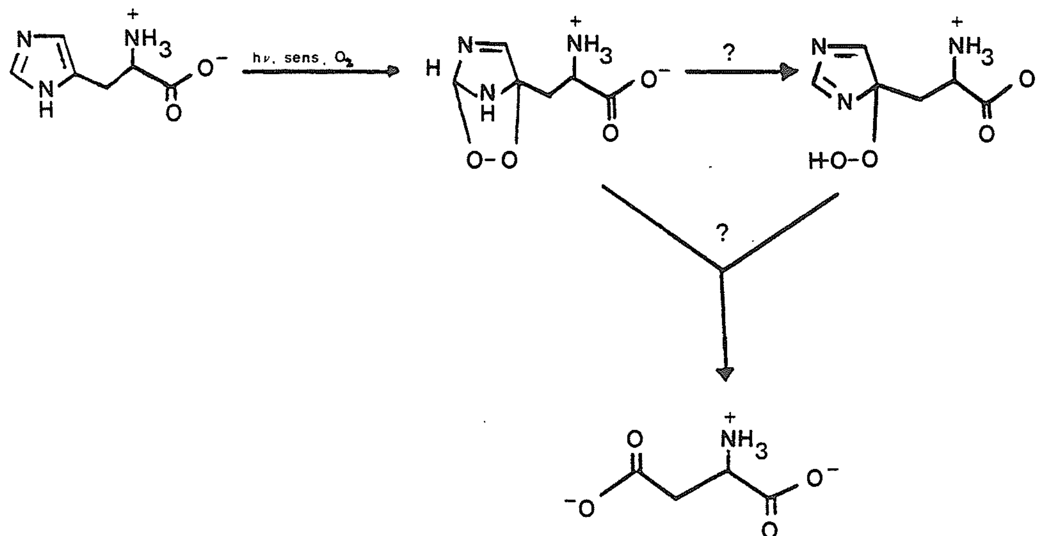
concomitant removal of or reduction in dissolved oxygen should have marked effects on a Type II process. This approach assumes that the oxygen levels can be significantly reduced in a range over which the reaction rate is dependent upon the oxygen concentration. Also assumed is that the removal of oxygen and addition of another gas will have no effects on the processes being studied other than to remove a possibly essential reactant.

Binding of a photosensitizer to an oxidation substrate could increase the probability of a Type I process occurring as well as increase the effectiveness of such a process. Alternatively, binding of the photosensitizer to the substrate could enhance the specificity of either a Type I or Type II reaction by serving to localize the site(s) of radical formation and/or singlet oxygen production. This type of interaction is easily monitored by a variety of methods including absorption and fluorescence techniques. The binding of a photosensitizer to a protein might cause alterations in the absorption and/or emission characteristics of either or both species (10). In conjunction with the results of other experiments such as those described above, interaction-dependent alterations of the spectral characteristics of the relevant molecules could aid in the determination of the existence and/or relative importance of Type I and Type II pathways in a photosensitized oxidation.

Photosensitized Oxidations: Susceptible Amino Acids

As stated previously the amino acids which are susceptible to photosensitization are cysteine, histidine, methionine, tryptophan and tyrosine (6,7,10,11). Arginine and lysine have also been reported to show reactivity at pH > 10 with high concentrations of some photosensitizers (10) and increasing methylation of the α-amino group of lysine has been shown to increase its susceptibility towards photosensitization (7). What follows is a brief summary of the conditions under which and possible mechanisms by which the sensitive amino acids react.

The deprotonated form of histidine reacts primarily by a Type II mechanism (10,13). Model studies suggest that cleavage of the imida-

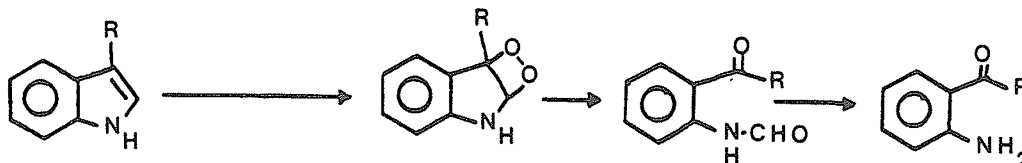


zole ring is followed by hydrolytic cleavage yielding aspartic acid (13). The protonated form of histidine is not susceptible to sensitized photooxidation (10,13).

Photooxidation of tyrosine proceeds by both Type I and Type II

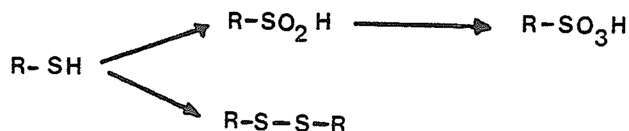
pathways (10). The anionic form of this residue is much more reactive towards sensitized photooxidation than the neutral form (10,13). The products of this reaction are not known but the reaction site is known to be the phenolic ring (10,13).

Photooxidation of tryptophan, which follows both Type I and Type II pathways, gives rise to a complex mixture of products (10,13).



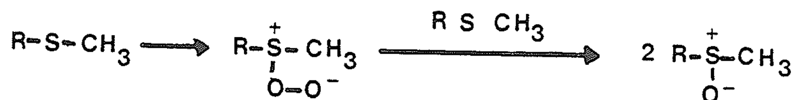
N-formylkynurenine may be the primary product but this species is apparently readily converted to a variety of other products (13).

Cysteine is slowly oxidized to cysteic acid *via* a Type II mechanism (13). Type I processes also occur but the products of these



reactions are not known (10,13).

The photosensitized oxidation of methionine proceeds by both Type I and Type II pathways (10,13). The product of Type II reactions is



usually the sulfoxide (10). Type I reactions yield a variety of products (10,13).

The rates and mechanisms of the photosensitized oxidations of all of the amino acids mentioned above are dependent on a variety of experimental conditions (7,10,13). Factors which may affect these reactions include the buffer pH and composition, choice of sensitizer

as well as the concentrations of oxygen, sensitizer and substrate. The alteration of one or more of these reaction conditions may result in profound rate changes and could also lead to a change of mechanism (from Type I to Type II or vice versa).

The photosensitized oxidation of susceptible residues in a protein may or may not differ from that of the free amino acids. Factors which do not influence the reactivity of free amino acids may be of great importance in protein studies. Blocking of the amino and carboxyl groups may alter the sensitivity of a residue to a Type I and/or Type II mechanism (7,10). The conformation of a protein may serve to protect otherwise susceptible residues and these protected residues may become progressively more exposed as other residues on the protein are oxidized (10).

In concluding this section a quotation from (10) seems appropriate:

> ... although a sensitized photooxidation process may superficially appear to be simple, it is usually an extremely difficult and time-consuming task to determine the detailed mechanism(s) involved.

Photosensitized Oxidations: Protein Studies

Studies of the photosensitized oxidation of proteins are numerous (for reviews see 6,10,13) and a comprehensive survey of this subject is beyond the scope of this review. The results of a few protein and whole

cell studies which involve the use of various photosensitizers are summarized below.

Trypsin/Xanthenes (25)

Various xanthenes (halogenated fluoresceins) were used to sensitize the photoinactivation of trypsin. The effects of azide and D_2O were not examined but red-shifts in the absorption maxima of several of the dyes studied were observed in the presence of trypsin. These authors determined that the photosensitizing efficiency and binding of the dyes to trypsin both increased with increasing number and atomic weight of the halogen substituents. A direct relationship was found between photoinactivation efficiency and the triplet state quantum yield of the dyes but no such relationship existed between photosensitizing efficiency and binding. The authors concluded that the ability of the xanthenes to photosensitize the inactivation of trypsin was primarily a function of the triplet state quantum yield and not of the ability of the dyes to bind to trypsin.

Lysozyme/Eosin Y (23)

In a more thorough study than that described above the effects of azide and D_2O on the eosin Y-sensitized photoinactivation of lysozyme were examined. Fluorescence quenching techniques were also employed. It was determined that singlet oxygen was the major inactivating species (primarily Type II pathway) when lysozyme was illuminated in the presence of free or bound eosin Y. A significant Type I process

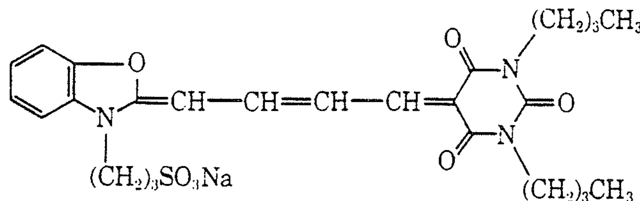
was also involved and its contribution to the inactivation varied with pH and the relative concentrations of sensitizer and enzyme. A tryptophanyl residue (Trp-108) was tentatively identified as the susceptible site. It was also suggested that destruction of this residue was a consequence of conformational changes brought about by singlet oxygen attack at other non-tryptophyl sites. The eosin Y binding site on the enzyme was also tentatively identified.

E. coli/Immobilized Rose Bengal (26)

This study was aimed at demonstrating the feasibility of using an immobilized photosensitizer to sensitize the photokilling of waterborne bacteria. The effects of D_2O, oxygen removal and light on the rose bengal-sensitized photoinactivation of *E. coli* were examined. It was concluded that singlet oxygen was at least partially responsible for the observed photoinactivation of *E. coli* in the presence of immobilized rose bengal and that this species attacked some site(s) on the surface of the cells. The nature of the sensitive sites was not investigated.

Photohemolysis/Rose Bengal and M-540 (27)

Merocyanine-540 (M-540; structure shown below) is currently being



Merocyanine-540

studied as a possible agent for the photodynamic treatment of leukemia and neuroblastomas. These investigations stemmed from the observation that this photosensitizer, while binding reversibly to excitable cells and blood cells, binds irreversibly to leukemic cells. Rose bengal is considered to be a prototypical singlet oxygen generator/sensitizer (27,28). Both M-540 and rose bengal were found to act as sensitizers in the photohemolysis system employed. The effects of azide on sensitized photohemolysis were identical for both the M-540- and rose bengal-sensitized reactions. The D_2O-dependent rate enhancement of the M-540-sensitized reaction was greater than that of the rose bengal-sensitized reaction. The authors concluded by proposing (see also 29,30) that both M-540 and rose bengal bind to the cell membranes after which they absorb light and subsequently generate the singlet oxygen which serves to rupture the membrane by its action at sensitive sites on the membrane. The nature of the sensitive site(s) was not investigated and the possible involvement of other excited intermediates in addition to that of singlet oxygen was not ruled out.

Lipoamide Dehydrogenase/Rose Bengal (24)

The effects of azide, D_2O and the electron acceptor $Fe(CN)_6^{3-}$ on the rose bengal-sensitized photoinactivation of lipoamide dehydrogenase were interpreted as indicating a mixed Type I - Type II mechanism for this process. The Type II pathway was observed to be responsible for the major proportion of the inactivation. It was inferred that dye-binding preceded photoinactivation from the hyperbolic nature of the inactivation rate dependence on sensitizer concentration. Earlier NMR studies had shown that histidine destruction was of primary importance in the sensitized photoinactivation (31) and this conclusion was further substantiated by amino acid analyses which showed that five histidine residues per subunit were destroyed in enzyme solutions which had been 90% photoinactivated. The effect of oxygen removal was not examined.

Photosensitizers: Psychological and Neurological Effects

In 1975 Feingold (32) proposed that hyperkinetic children were genetically predisposed to be sensitive to artificial food additives. He claimed to have had dramatic success in alleviating the behavioral symptoms of this and other disorders by placing patients on a diet devoid of such additives. Among the implicated chemicals was the xanthene erythrosin (erythrosin B, erythrosine, F.D.&C. Red No.3). Ensuing studies have seemed to suggest that within the population of hyperkinetic children there exists a sub-group who are sensitive to

such food additives and who respond favorably to treatments such as that described above (33). Understandably, the results of human studies aimed at answering such questions are difficult to interpret as the complexity of the possibly significant variables is overwhelming.

In contrast to human behavioral studies, *in vitro* experiments with erythrosin and a variety of biological preparations have shown conclusively that this compound does significantly affect isolated systems of neurological importance. The systems affected include synaptosomal dopamine and glutamic acid uptake (34), Ca^{2+}:Mg^{2+}-ATPase in sarcoplasmic reticulum (35), ouabain binding in heart and brain tissue of rats (36) as well as a variety of natural and artificial membranes (e.g.26,27,29,37,38). In most of these studies the light-dependency of the observed phenomena were examined.

A 1983 publication of the American Food and Drug Administration (FDA) reported that no conclusive evidence had, as of that date, been presented which implicated the chronic ingestion of erythrosin as a cause of adverse neurological effects (39) despite an FDA-estimated per capita intake of 34 mg/day. Pooler and Valenzeno (40) had earlier pointed out that the toxicity and carcinogenicity testing protocols of the FDA did not include the examination of the light-dependent effects of the chemicals tested. This lack was not addressed in the 1983 FDA publication (39).

In addition to their use in foodstuffs, drugs and cosmetics, erythrosin and other photosensitizing xanthenes have been found to be either useful or potentially useful in retinal angiography (41,42). Given the unknown metabolism of these compounds upon ingestion or injection (39) and their significant phototoxic potential the intake of

these compounds in any manner seems to this author to be highly undesirable. Of relevance to this line of thought are the observations that: 1) retinal angiography patients turn visibly green for several hours following the injection of 5 mL of a 10% fluorescein solution (42); 2) sunlight of exciting wavelengths (for xanthenes) does penetrate human skin to the dermal capillaries (19); and finally, 3) artificial light of comparable brightness to that of sunlight does elicit erythrosin-sensitized effects at sub-micromolar dye concentrations (35).

It seems appropriate to conclude this section with a quotation from a previously cited publication (40) in which the authors make a dramatic plea to the biomedical community:

> ... we are doubly concerned about the risks involved when photosensitizing substances are introduced in the human body and urge that all toxicity tests of these materials include phototoxicity tests as well.

Summary

Several uses of photosensitizers have been described. Among these uses are the following:

i) reagents for the modification of proteins as well as cell membranes.

ii) reagents for mechanistic studies of the photosensitized oxidation of various substrates.

iii) therapeutic agents in the photodynamic treatment of leukemia and neuroblastomas.

iv) colorants in foods, drugs and cosmetics.

Current research is also aimed at developing the potential of a variety of photosensitizers to act as light-activated insecticides and herbicides (15).

Studies of the light-dependent effects of the xanthenes, particularly erythrosin, were initiated in this laboratory for two reasons. Firstly, as a mild and possibly site-specific labelling or modification technique it seemed reasonable to expect that some information regarding the nature of catalytically important groups in the neurologically important enzyme acetylcholinesterase might be gained (43,44). Secondly, in light of the paucity of detailed mechanistic studies on protein photosensitization and the apparently complex mechanism(s) involved in the erythrosin-sensitized photoinactivation of AChE we decided to perform an in-depth examination of this process.

MATERIALS

Materials	Supplier
crude 11s *E. electricus* AChE (Type VI-S) Acetylthiocholine 5,5'-Dithiobis-(2-nitrobenzoic acid) Electrophoresis standards (MW-SDS-200) d-Tubocurarine Tris(hydroxymethyl)aminomethane Decamethonium bromide	Sigma Chemical Company St. Louis, Missouri, U.S.A.
Dithiothreitol Sodium dodecyl sulfate Acrylamide Methylene-bis-acrylamide Ammonium persulfate N,N,N',N'-tetramethylethylenediamine Bio-Gel P-6DG columns	Bio-Rad Laboratories (Canada) Ltd. Mississauga, Ontario, Canada
DCl, NaOD, D_3PO_4 (Gold Label, 99+ % atom D) D_2O (Gold Label, 99.8% atom D)	Aldrich Chemical Company Milwaukee, Wisconsin, U.S.A.

Materials	Supplier
3-ft. double tube fluorescent fixtures 3-ft. cool white fluorescent tubes (Sylvania/Lifeline:F30T12/D/RS)	Robinson Lighting Winnipeg, Manitoba, Canada
Argon (99.995% min. purity)	Matheson Gas Products Whitby, Ontario, Canada
Edrophonium chloride (Lot # 044033)	Hoffman - La Roche Inc. Nutley, New Jersey, U.S.A.
Amicon PM10 membranes	Amicon Canada Ltd. Oakville, Ontario, Canada
N-methyl-7-hydroxyquinoline (M7H) N-methyl-7-(dimethylcarbamoxy) quinolinium iodide (M7C)	Eastman Kodak Co. Rochester, New York, U.S.A.

Materials	Supplier
Erythrosin B (Gurr)	BDH Chemicals
Fluorescein (Gurr)	Toronto, Ontario,
Eosin Y (Harleco)	Canada
Eosin B (Harleco)	
Rose bengal (BDH)	
Ethanol	Fisher Scientific
Sucrose	Winnipeg, Manitoba,
Glycine	Canada
Hydrochloric acid	
Sodium hydroxide	
Sodium chloride	
Phosphorus pentoxide	
Sodium azide	
Monobasic and dibasic sodium phosphate	
Volumetric pipette tips	
Polystyrene cuvettes	

Buffers and Reagents

1) Ellman assay buffer : 0.10 M NaH_2PO_4, pH 8.0 in H_2O.

2) Equilibration buffer (EQB) : 0.010 M NaH_2PO_4, 0.10 M NaCl, pH 7.4 in H_2O.

3) 5,5'-dithiobis-(-2-nitrobenzoic acid) : 0.010 M DTNB in Ellman assay buffer. DTNB was recrystallized from ethanol prior to use as described in (50).

4) Acetylthiocholine (ASCh) : 0.0764 M in H_2O, prepared fresh every 4 days.

5) Dye solutions : these were prepared in Ellman assay buffer such that 5 μL (usually) in a total volume of 3.055 mL (usually) would result in a dye concentration of 5 or 10 μM. The concentrations of these stock solutions were determined spectrophotometrically.

All buffers used in these studies were prepared from glass-distilled deionized water and then ultrafiltered on a Millipore Ultrafiltration Apparatus with Millipore Type HA (0.45 μm pores) filters.

METHODS

Purification of *E. electricus* AChE (E.C.3.1.1.7)

Type VI-S AChE from Sigma was purified by affinity chromatography according to the method of Dudai *et al.* (45) with minor modifications (as described in 46). 2-4 vials of crude enzyme were dissolved in 2-4 mL of EQB and applied to the affinity column (3 mL bed volume; preparation of column described in 46). The column was then washed with EQB until the eluate had the same A_{280} as the eluent. The AChE was then eluted with EQB containing 0.010 M DMB. The active fractions were pooled and applied to a CM-50 column equilibrated with EQB (50 mL bed volume). This column was then allowed to run dry. Effective removal of DMB was checked by performing serial dilutions of the final AChE solution and checking for increases in total activity of the original solution with increasing dilution. In some instances the final AChE solution (post CM-50) was concentrated on an Amicon ultrafiltration device equipped with Amicon PM10 membranes. This final solution is referred to as 'concentrated AChE' throughout the remainder of this paper. Small aliquots of concentrated AChE were stored at -20 °C.

Routine Assays for AChE Activity

AChE activity was routinely determined by the method of Ellman *et al.* (47). The assay conditions were 0.10 M NaH_2PO_4, 0.49 mM ASCh and 0.32 mM DTNB. The production of the thionitrobenzoate anion generated by the reaction of DTNB with the hydrolysis product thiocholine was

monitored at 412 nm ($\varepsilon_{M\,412} = 1.36 \times 10^4\ M^{-1}cm^{-1}$) on a Beckman Model 25 Spectrophotometer or a Hewlett Packard HP8452A Spectrophotometer interfaced with a Mind (IBM XT compatible) computer. AChE solutions were diluted so that 5 μL of enzyme solution in a total reaction volume of 3.125 mL gave a slope of less than 1.0 A_{412} units min^{-1}.

Active Site Determination

AChE active site concentrations were determined from the reaction of AChE with the fluorogenic carbamoylating agent M7C as described in (46). These determinations were performed on a Perkin-Elmer MPF-44 Fluorescence Spectrophotometer. Excitation and emission wavelengths were 408 nm and 504 nm respectively. Both slits were 11 nm and the M7C concentration was 2.0 μM. The standard curve typically spanned an M7H concentration range of 1.25 nM - 23.80 nM.

Electrophoresis

Rod gel electrophoresis using 7.5 % gels with 3.8 % stacking gels (200 μL) was by the method of Laemmli (48) as described by Hames and Rickwood (49). Electrophoresis was carried out with a Bio-Rad Model 300B Rod Gel Apparatus equipped with a Model 400 Power Supply. Current was constant at 3 mA/rod and voltage was set at 'full' (> 500 V) for the duration of the experiment (typically 4 hr). The rods were cooled with cold tap water throughout the experiment. Gels were stained with 0.1 % Coomassie blue in water:methanol:acetic acid (5:5:2 by volume) and diffusion destained in water:methanol:acetic acid (6:3:1 by

volume). Gels were stored in a 7 % acetic acid, 5 % methanol solution at 4 °C. Sample volumes ranged from 5-10 μL for standards to 50-150 μL for AChE solutions. All samples were immersed in boiling water for five minutes prior to electrophoresis. Buffer compositions were:

Stacking gel : 0.125 M Tris, 0.1 % SDS, pH 6.8.

Resolving gel : 0.375 M Tris, 0.1 % SDS, pH 8.8.

Reservoirs : 0.025 M Tris, 0.192 M glycine, 0.1 % SDS, pH 8.3.

Sample : 0.0625 M Tris, 2 % SDS, ±0.05 M DTT, 10 % sucrose, pH 6.8.

<u>Densitometry</u>

A densitometric scan of a gel containing Coomassie blue - stained reduced AChE was obtained on a LKB 2202 Ultroscan Laser Densitometer interfaced with an LKB 2220 Recording Integrator. The following instrument settings were used:

<u>densitometer</u>	scan speed : 40 mm/min
	absorbance range : 2
<u>integrator</u>	attenuation : 10
	chart speed : 5 cm/min
	threshold : 10
	peak width : .04
	area rejection : 0

Photoinactivation Studies

Photoinactivations were carried out by placing a polystyrene cuvet containing 3.0 mL of Ellman buffer, 30-50 μL concentrated AChE solution and a small aliquot of dye solution (5-25 μL) in between 2 banks of 2-3 ft. 40W fluorescent tubes which were 10 cm on either side of the cuvet. The cuvets were placed in the same position for all photoinactivations. For studies of the kinetics of photoinactivation duplicate 30 μL samples were withdrawn at the appropriate times and assayed for AChE activity (Ellman assay).

The final concentration of dye (and inhibitor if present - see below) in the Ellman assay was such that inhibition of active AChE was negligible. Irradiation of the enzyme (up to 20 min) in the absence of dye had no effect on enzyme activity and dark controls showed that no irreversible inhibition of AChE occurred under conditions which were otherwise identical to those of the photoinactivation experiments. Photobleaching of the dyes in the absence of AChE was minimal (less than 10% in 20 min) at dye concentrations at or above 5 μM but increased at lower concentrations. The irradiation time was 5-10 min in most experiments.

For anaerobic photoinactivation studies water-saturated argon was bubbled through 3 mL of Ellman buffer in a polystyrene cuvet, at the fastest rate possible which would not cause the buffer to spill out of the cuvet, for at least 30 min. Concentrated enzyme and dye were then added and this mixture was kept under a blanket of argon for the duration of the photoinactivation. In the aerobic studies all solutions used were in equilibrium with the ambient air. All Ellman assays

were performed aerobically and all of these experiments were carried out at room temperature (22 ± 2 °C).

During the initial stages of the anaerobic studies oxygen levels were checked to see if the argon treatment did effectively remove oxygen. A Rank Brothers (Cambridge, England) silver/platinum oxygen electrode equipped with High Sensitivity Teflon Membranes (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) was used to measure oxygen levels. The treatment was found to reduce the oxygen levels of the buffer to below the detection limits of this instrument within several minutes. Later it was determined that the photoinactivation rates increased with the time of bubbling up to 30 min after which the rates showed no increase with increasing bubbling time. Consequently, for the anaerobic experiments argon was always bubbled through the 3 mL of Ellman buffer for at least 30 min prior to the start of a photoinactivation.

The buffers for the pH studies were prepared by combining appropriate volumes of 0.5 M solutions of NaH_2PO_4 and Na_2HPO_4 adjusted to pH 6.5 and 9.5 respectively, and diluting with water to a final phosphate concentration of 0.10 M. Although the buffering capacity of some of these solutions was minimal it was determined that there was no pH change when a photoinactivation was carried out in water. This suggested that buffering capacity was not critical to these experiments.

When AChE inhibitors (edrophonium, d-tubocurarine) were included in the photoinactivation experiment a small aliquot (5-10 μL) of a concentrated solution was added to the Ellman buffer prior to the addition of AChE. In the anaerobic experiments this addition was done

prior to the argon treatment.

Photoinactivations performed in D_2O-based buffer were identical to those in H_2O-based buffer except that the buffer was prepared from D_2O, DCl, NaOD and 0.10 M D_3PO_4. Controls showed that in the absence dye or irradiation the stability of AChE was identical in the D_2O- and H_2O-based buffers under both aerobic and anaerobic conditions.

Azide-containing buffer was prepared separately for those experiments which involved the use of azide. Controls showed that azide itself had no effect on the stability of AChE under any of the reaction conditions employed in these studies.

Dye-binding Studies

Binding of erythrosin or rose bengal to AChE was examined by the addition of dye solution to 2 mL of concentrated enzyme and monitoring changes in the absorption spectra of the dyes. Spectra were recorded on a Beckman Model 25 Spectrophotometer.

Quenching of protein fluorescence was monitored on a Perkin-Elmer MPF-44 Fluorescence Spectrophotometer. 5-10 μL aliquots of dye solution (in EQB) were added to 2 mL of concentrated AChE. Excitation and emission slits were 3.6 nm and 8.0 nm respectively. Excitation was at 280 nm and emission was monitored at 340 nm. Fluorescence intensities were expressed relative to a tryptophan solution, which had the same 280 nm absorbance as the enzyme solution, to which equivalent additions of dye solution were made. Dilution corrections were made.

Calculation of Apparent Dye-AChE Dissociation Constants

Apparent dissociation constants of the dye-AChE complexes were calculated from the kinetic and fluorescence-quenching data using the equations shown below.

kinetic data $$1/k_{obs.} = 1/[dye] \cdot K_d/k_{max.} + 1/k_{max.}$$

where $k_{obs.}$ = the observed first-order rate constant

$k_{max.}$ = the maximum possible value of $k_{obs.}$

K_d = the apparent dye-AChE dissociation constant

[dye] = the dye concentration at which $k_{obs.}$ was observed

fluorescence data $$1/F = 1/[dye] \cdot K_d/F_{max.} + 1/F_{max.}$$

where F = the observed relative fluorescence

$F_{max.}$ = the maximum possible value of F

K_d = the apparent dye-AChE dissociation constant

[dye] = the dye concentration at which F was observed

Double-reciprocal plots were constructed from the relevant data and K_d, $k_{max.}$ and $F_{max.}$ values were calculated from the slopes and y-intercepts of these plots.

Amino Acid Analyses

Samples for amino acid analysis were prepared in this lab and sent to the Protein Sequencing Facility at the University of Calgary for hydrolysis and analysis. Inhibitor and/or dye (both 10 μM) was added to 3.0 mL of concentrated AChE and irradiated for 10 min (approximately 10% activity remaining). Protein was then separated from dye/inhibitor and simultaneously desalted on Bio-Rad P-6DG columns. Anaerobic samples were prepared in the same manner except that prior to photoinactivation the AChE solution was diluted with 4 volumes of argon-saturated EQB and then concentrated under argon on an Amicon ultrafiltration apparatus. Anaerobic samples were kept in an argon atmosphere during photoinactivation (7 min, approximately 10% activity remaining) and application to the desalting columns.

Samples for tryptophan analysis were hydrolyzed in 6N HCl/4% thioglycolic acid *in vacuo* at 110 °C for 24 hours as described in (51). Cysteine analysis involved performic acid oxidation and HCl hydrolysis according to (52). The remaining samples were hydrolyzed in 6N HCl, 0.1% phenol and 0.1% thioglycolic acid *in vacuo* at 110 °C for 24 hr according to (53). Amino acids were analyzed on a Beckman 6300 Amino Acid Analyzer with ninhydrin detection.

RESULTS & DISCUSSION

Purification of AChE

The concentrated AChE solutions used for the experiments described in this paper had specific activities ranging from 4000 to 6200 U/mg. Concentrations ranged from 87 to 162 μg/mL. In some cases active site concentrations were determined using the M7C assay. Samples of the highest specific activity were used in experiments involving amino acid analyses.

The AChE inhibitor DMB was always completely removed from the enzyme solution by chromatography on CM-50. There was no loss of activity in the concentrated AChE solutions upon storage at -20 °C for extended periods of time (up to 2 years). Repeated freezing and thawing (10-15 times) did result in small losses (5-10%) of AChE activity.

The results of SDS-PAGE of concentrated AChE solutions in the presence and absence of DTT are shown in Figure 3. Densitometric scans of similar gels are shown in Figure 4. The molecular weights of the standards used for the calibration plot (R_f *vs.* log molecular weight; data not shown) ranged from 45 to 200 kilodaltons (kD). The weights calculated for the AChE tetramer (from extrapolation), dimer and monomer were 258 kD, 147 kD and 51 kD respectively. These results are in agreement with those of Rosenberry (54) although the weight of the monomer is slightly low (51kD vs 70kD). This discrepancy could be due to the different electrophoretic procedure used and to proteolysis of the enzyme during isolation. Figures 3 and 4 also suggest that although

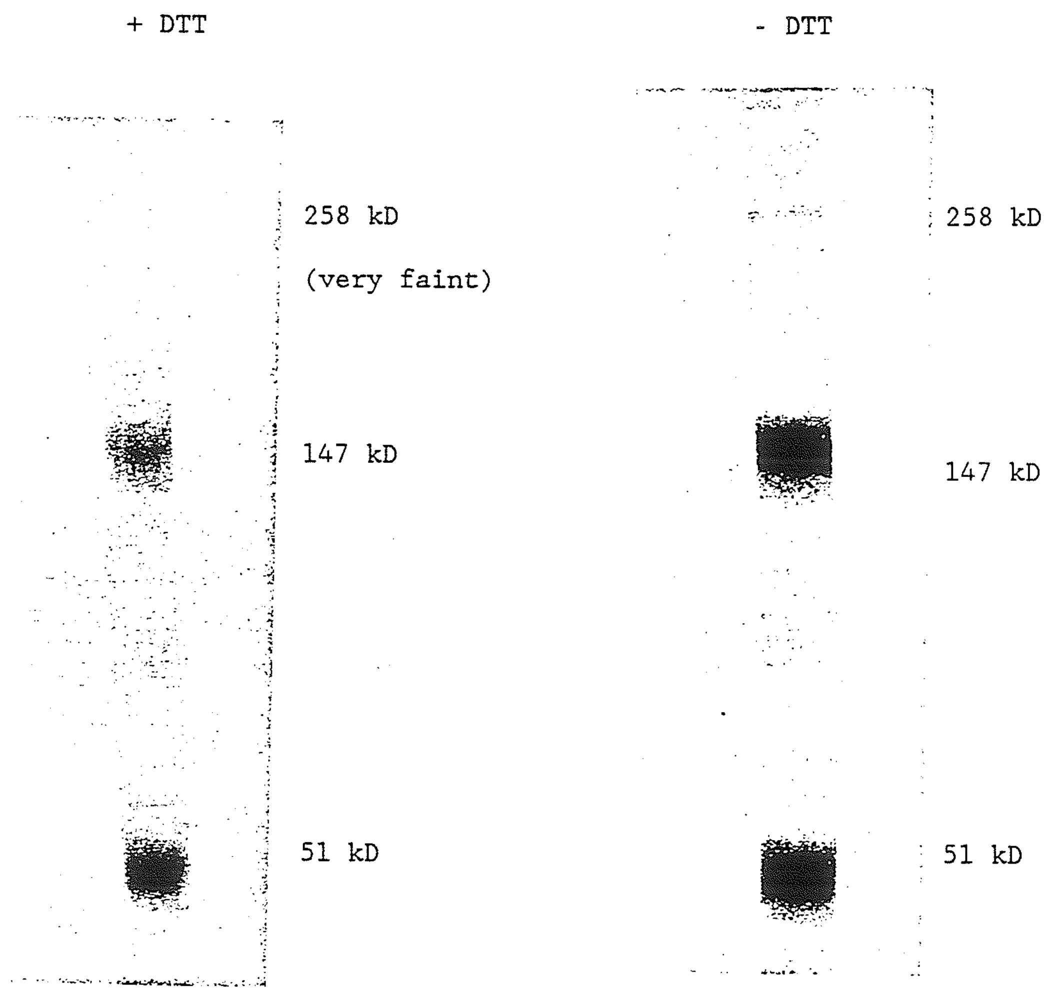


Figure 3. SDS gels of Coomassie Blue-stained AChE prepared in the presence and absence of dithiothreitol.

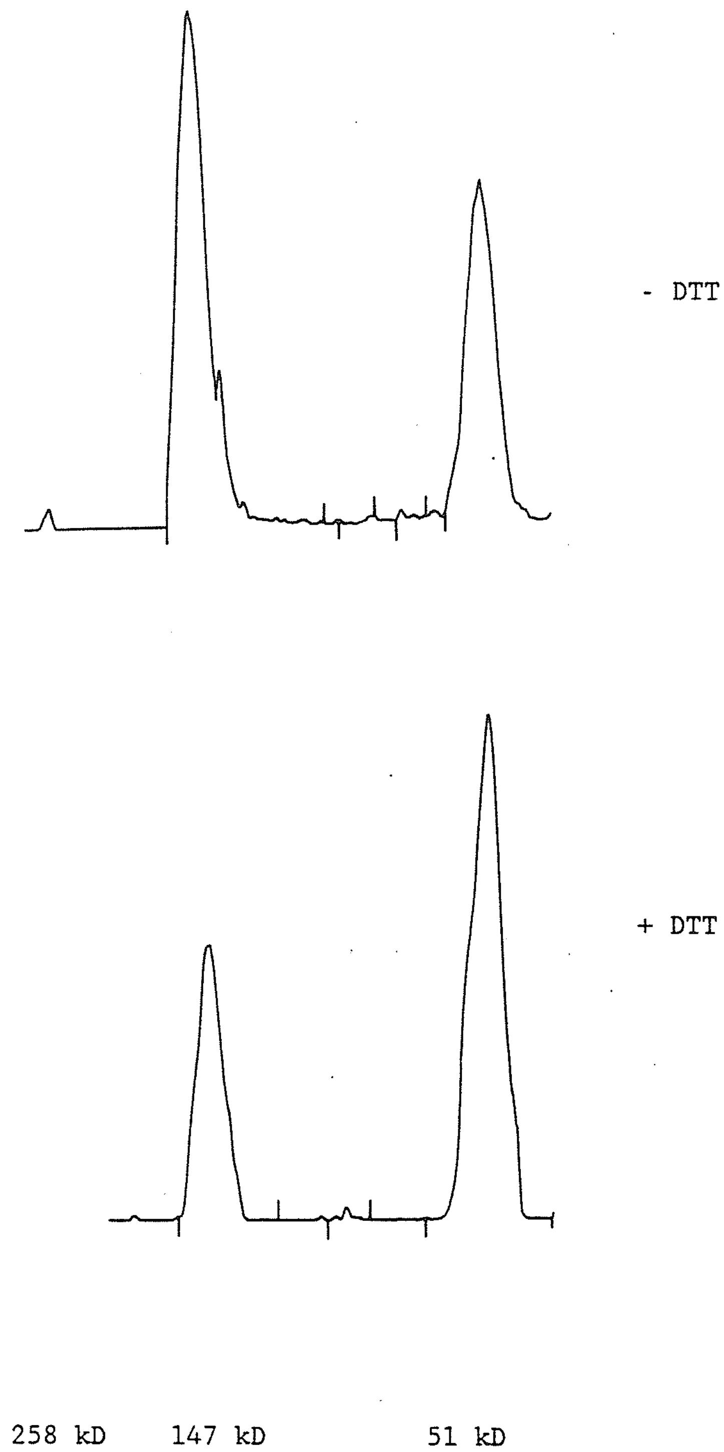


Figure 4. Densitometric scans of SDS gels containing AChE prepared in the presence and absence of dithiothreitol.

reduction occurred in the presence of DTT the treatment used did not effect complete reduction of the enzyme. These results do show that there was no significant amount of Coomassie Blue-stainable contamination in our AChE preparations.

Sensitized Photoinactivation: Xanthenes

The xanthene dyes rose bengal, erythrosin, eosin Y, eosin B and fluorescein all sensitize the photoinactivation of AChE. The data presented in Figure 5 show that the effectiveness of these dyes as photosensitizers parallels the triplet state quantum yields of the dyes (see Table 1) with rose bengal being the most effective and fluorescein the least. Since saturation behavior was suggested by the data shown in Figure 5 a more detailed study of the concentration dependence of erythrosin-sensitized photoinactivation was carried out.

Sensitized Photoinactivation: Erythrosin

Erythrosin-sensitized photoinactivation of AChE exhibited first-order kinetics over the dye concentration range 1-50 μM (Figure 6). The observed rate constants increased hyperbolically with increasing erythrosin concentration under both aerobic and anaerobic conditions (Figure 7A) although the rate constants were three to five times greater under anaerobic conditions. The apparent saturation behavior was maintained when the experiments were carried out in 0.10 cm cuvets (*vs.* the 1.0 cm path length of the cuvettes used in most of the experiments; data not shown) and was therefore not due to the high

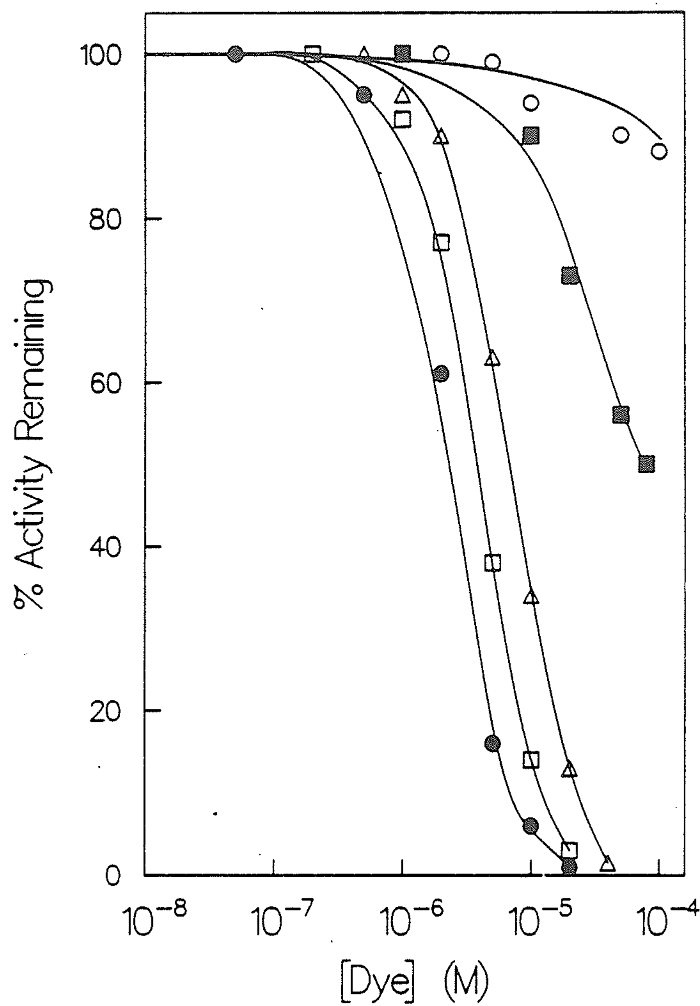


Figure 5. Photoinactivation of AChE by various xanthene dyes. Irradiation was for 10 min in all cases; ● = rose bengal, □ = erythrosin, △ = eosin Y, ■ = eosin B, ○ = fluorescein.

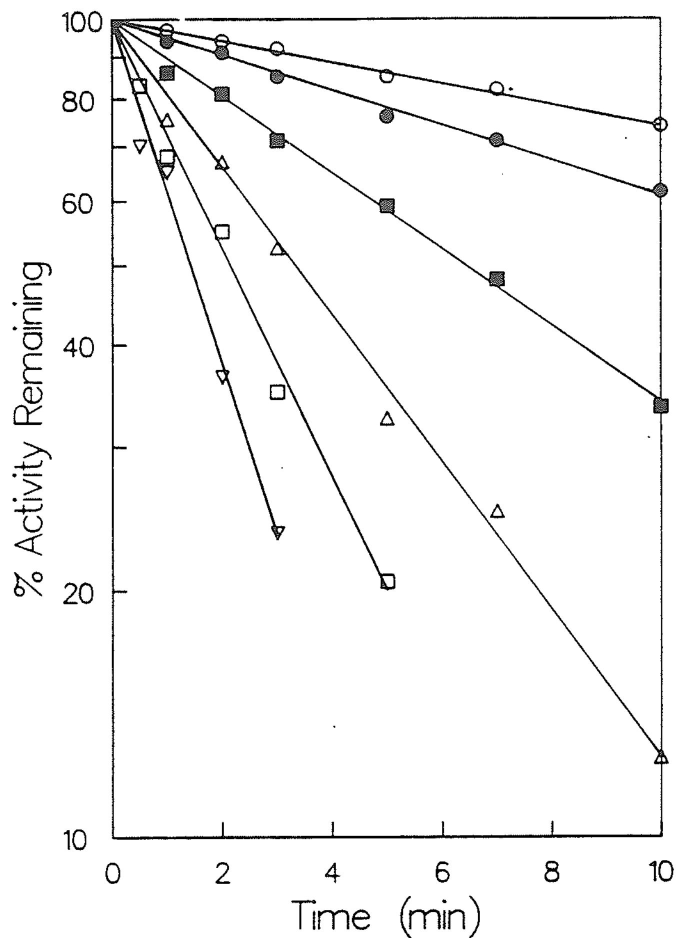


Figure 6. First-order Plots of the Kinetics of Erythrosin-sensitized Photoinactivation of AChE (aerobic). Dye concentrations used were 1(○), 2(●), 5(■), 10(△), 20(□) and 50(▽) μM.

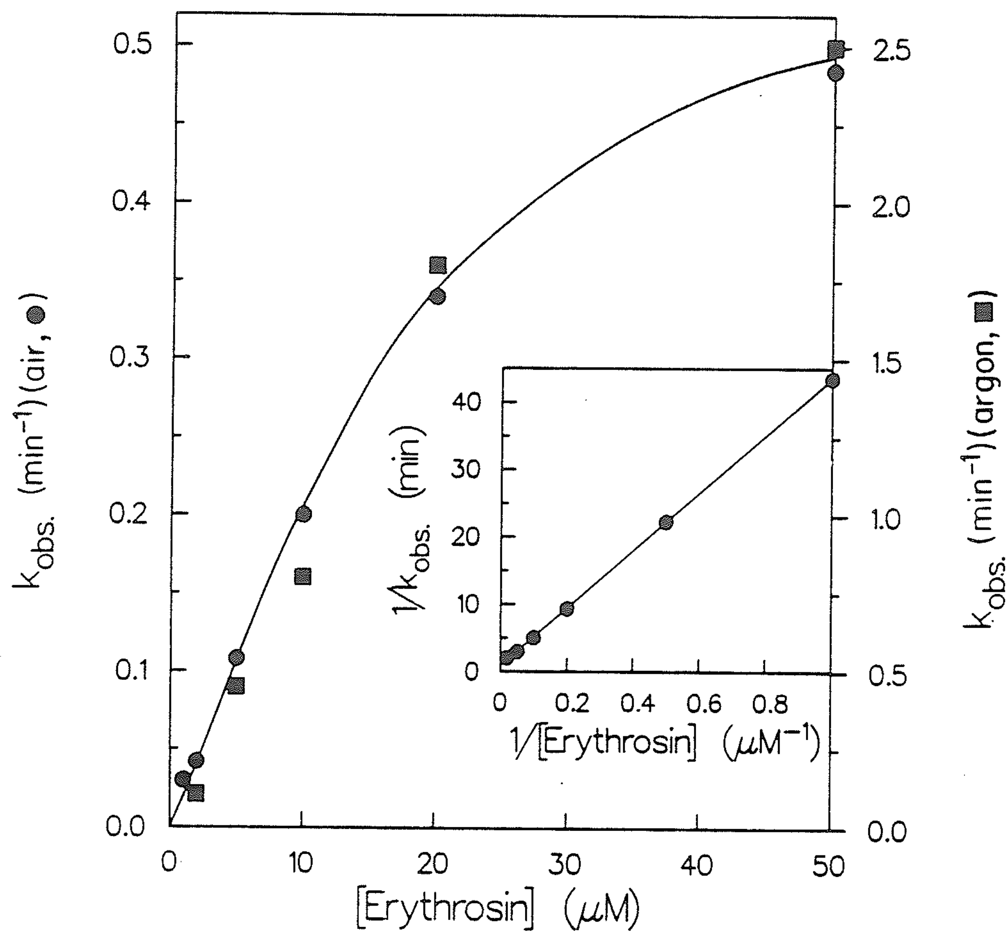


Figure 7. (A) The effect of erythrosin concentration on the observed first-order rate constant ($k_{obs.}$) of erythrosin-sensitized photoinactivation of AChE under aerobic (●) and anaerobic (■) conditions. (B) Double-reciprocal plot of data from A under aerobic conditions.

light absorbance of the dye solutions, at least up to a dye concentration of 50 μM. Accurate rate determinations could not be carried out at erythrosin concentrations exceeding 50 μM as the rates of photoinactivation were very high.

An apparent dye-enzyme dissociation constant of 48 μM was calculated from a double-reciprocal plot (Figure 7B) of the aerobic kinetic data shown in Figure 7A. Experiments involving the inclusion of erythrosin in the Ellman assay were performed (data not shown) to see if erythrosin inhibited AChE. Erythrosin behaved as a weakly competitive inhibitor with an approximate K_I of 120 μM.

Although the data presented so far may be interpreted as being indicative of a photosensitization mechanism wherein dye-binding to AChE precedes photoinactivation this has not been conclusively established. The saturation behavior is apparent but may or may not be a reflection of such a mechanism.

Erythrosin-sensitized Photoinactivation: Azide, Argon and D_2O

The fact that erythrosin-sensitized photoinactivation of AChE occurs under both aerobic and anaerobic conditions (see Figure 7B) suggests that both Type I and Type II (see Figure 1) pathways contribute to the photosensitization process. Table 2 lists the observed rate constants of erythrosin-sensitized photoinactivation in H_2O and D_2O under both aerobic and anaerobic conditions as well as the effects of azide on these rates.

Under aerobic conditions the rate constant in D_2O is eight times greater than that obtained in H_2O. 10 mM azide caused a reduction of

Reaction conditions	$k_{obs.}$ (min^{-1})	
	H_2O	D_2O
Air-saturated	0.18	1.48
Air-saturated + 10 mM azide	0.06	0.06
Argon-saturated	0.62	1.46
Argon-saturated + 10 mM azide	0.55	0.34

Table 2. The effects of D_2O and azide on the first-order rate constants of photoinactivation of AChE. pH, pD = 8.0; [erythrosin] = 5 μM; [AChE] = 1.07 μM in sites.

the rate constant in H_2O to one-third of its value in the absence of azide. The observed rate constant was dependent on azide concentration up to 10 mM but was independent of higher azide concentrations (Figure 8). This residual photoinactivation may represent the contribution of a Type I pathway under aerobic conditions. In D_2O 10 mM azide caused a twenty-five-fold reduction in the rate constant. This reduction is much higher than the ten-fold reduction commonly expected (21) based upon singlet oxygen quenching. The similarity of the rates in H_2O and D_2O in the presence of azide may reflect the effective quenching of all the singlet oxygen in either solvent such that the residual photoinactivation is due to a Type I process which is unaffected by the solvent change under aerobic conditions.

Under anaerobic conditions the photoinactivation rate constant in H_2O was approximately four times greater than the corresponding aerobic value and the effect of 10 mM azide was minimal (Table 2). Using D_2O as a solvent caused a two-fold rate increase and 10 mM azide reduced this rate constant by 75%. These results and those presented in the preceding paragraph may indicate that oxygen removal was incomplete. This could explain the similarity of the rate constants in D_2O under both aerobic and anaerobic conditions but does not explain the effect of azide on the anaerobic reaction in D_2O. The data may also indicate that the Type I process is accelerated in D_2O.

The results of this and the preceding section support the hypothesis that the erythrosin-sensitized photoinactivation of AChE proceeds predominantly by a Type II mechanism under aerobic conditions. Several factors which prompted us to explore the possibility that this process may proceed by an alternative, competing Type I pathway are

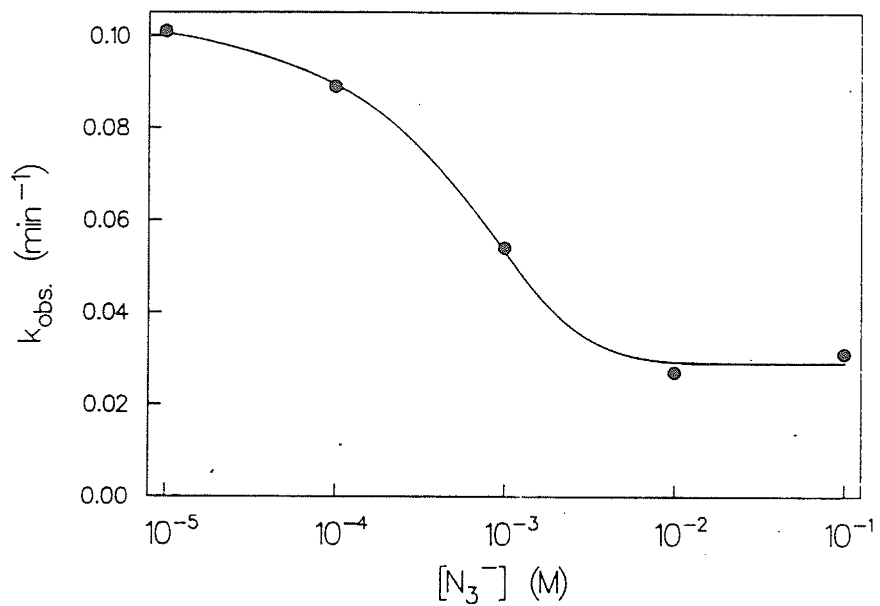


Figure 8. Quenching of the erythrosin-sensitized photoinactivation of AChE by azide under aerobic conditions.

listed below.

i) The rate constants of erythrosin-sensitized photoinactivation increase hyperbolically under aerobic and anaerobic conditions (Figure 7A). This is suggestive of the formation of a dye-enzyme complex which might favor or promote a Type I pathway.

ii) Azide quenching of both the aerobic and anaerobic photoinactivation was incomplete over a wide range of azide concentrations.

iii) The D_2O-dependent rate enhancement was less than that expected for a purely Type II process.

iv) Removal of oxygen resulted in a rate increase rather than the decrease expected for a purely Type II process.

v) Azide had little or no effect on the rate constant of anaerobic photoinactivation in an H_2O-based buffer.

Collectively these results indicate that the erythrosin-sensitized photoinactivation of AChE proceeds by a mixed Type I - Type II mechanism with the Type I pathway dominant under anaerobic conditions and the Type II pathway predominating under aerobic conditions (ii-14; iii-10, 16-19; v-18,19,21,22). The relatively small D_2O- and azide-effects observed under anaerobic conditions may be indicative of secondary effects of D_2O and azide on the photoinactivation process or may simply reflect the incomplete removal of oxygen by argon gassing.

Spectroscopic Studies

Evidence for the formation of an erythrosin-AChE complex was obtained using absorption and fluorescence techniques. Figure 9A shows the visible absorption spectrum of 0.63 μM erythrosin in the presence and absence of 1.07 μM active AChE subunits. The 3 nm red shift in the absorption maximum and the changes in the shape of the spectrum, brought about by the presence of the enzyme, are both indicative of complex formation.

During the preparation of photoinactivated samples for amino acid analysis we noticed that although the protein fractions were separated from most of the dye on Bio-Gel P-6DG columns they retained a slight pink coloration. Figure 9B shows that the absorption maximum of this material is red-shifted 7 nm from that of the dye alone.

Quenching of protein fluorescence at 340 nm over the concentration range 5-65 μM (Figure 10, Table 3) allowed estimation of the apparent dye-enzyme dissociation constant from double-reciprocal plots of the data (Figure 11). These values were 21 μM for rose bengal and 33 μM for erythrosin - a value comparable to that obtained from the kinetic data. AChE remained fully active throughout these titrations but exposure to bright light led to irreversible inactivation and loss of protein emission (see Figure 10, Table 3). Dye binding under anaerobic conditions was not studied.

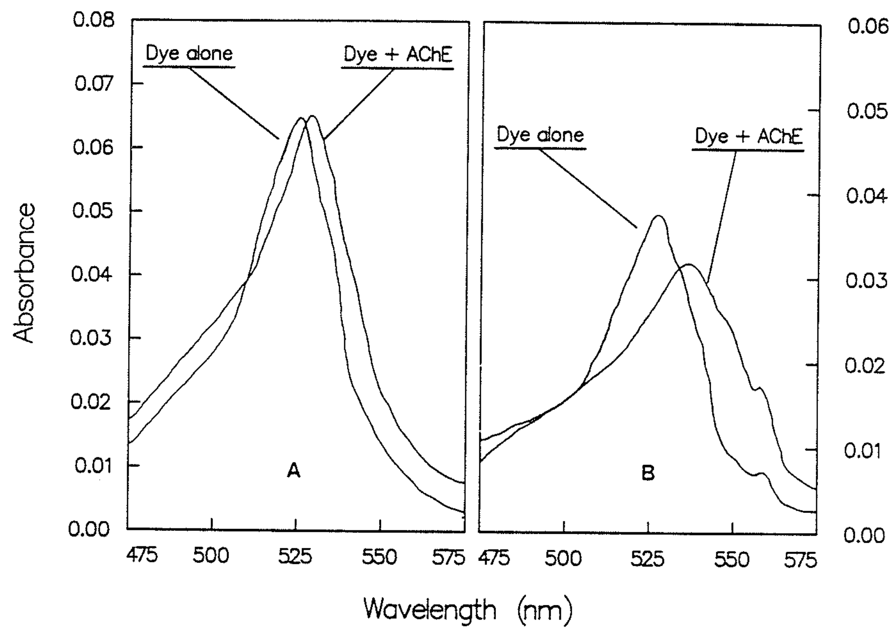


Figure 9. Absorption spectra of erythrosin in the presence and absence of AChE. (A) Before photoinactivation (1.07 μM AChE sites + 0.63 μM dye). (B) After photoinactivation and gel filtration on G-75 (concentrations unknown).

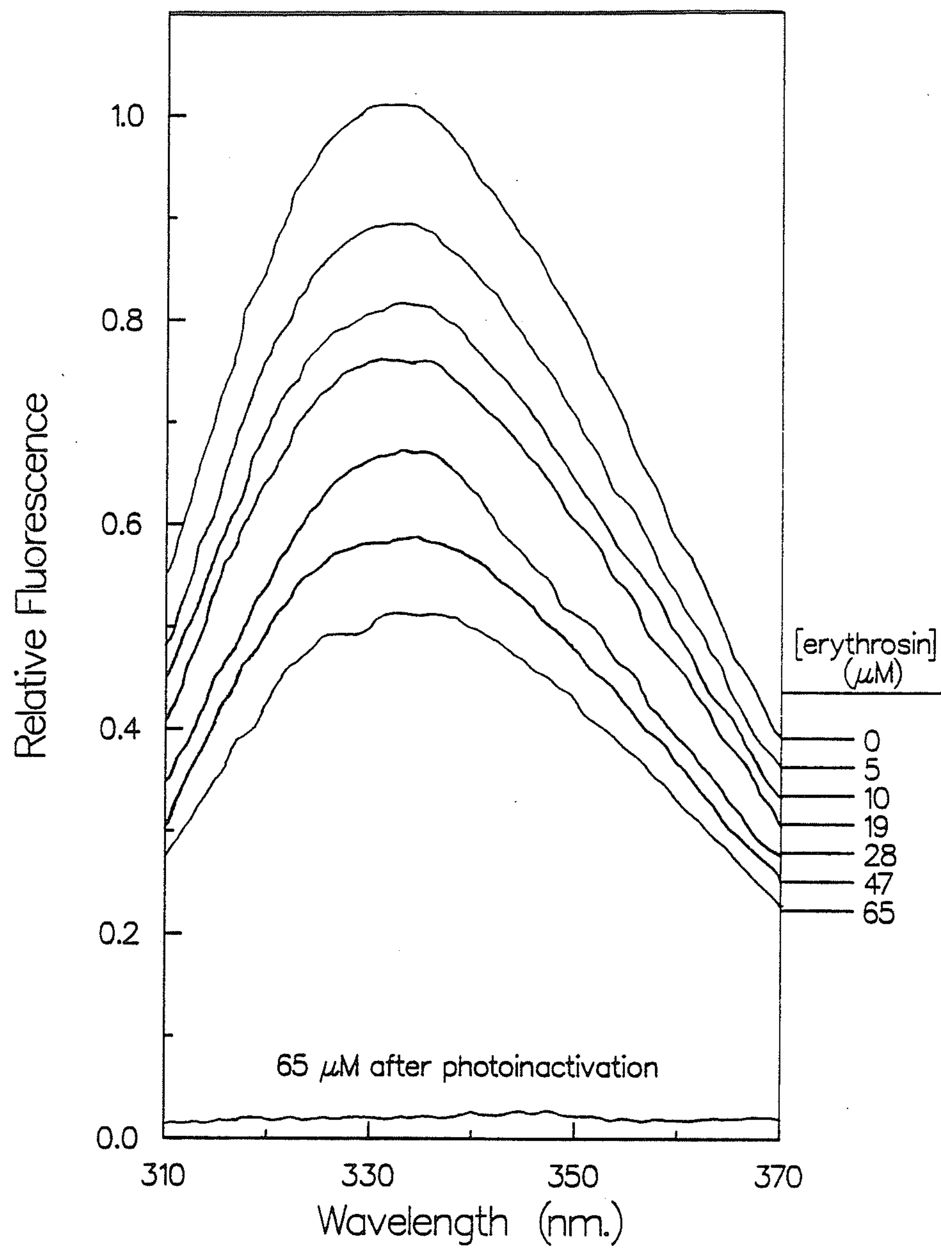


Figure 10. Quenching of AChE fluorescence emission by erythrosin.

[Erythrosin] (μM)	Relative fluorescence
Dark	
0.0	1.00
5.0	0.89
10.0	0.81
19.0	0.76
28.0	0.66
47.0	0.58
65.0	0.51
After irradiation	
65.0	0.00

Table 3. Quenching of AChE fluorescence by erythrosin ([AChE] = 1.07 μM in sites).

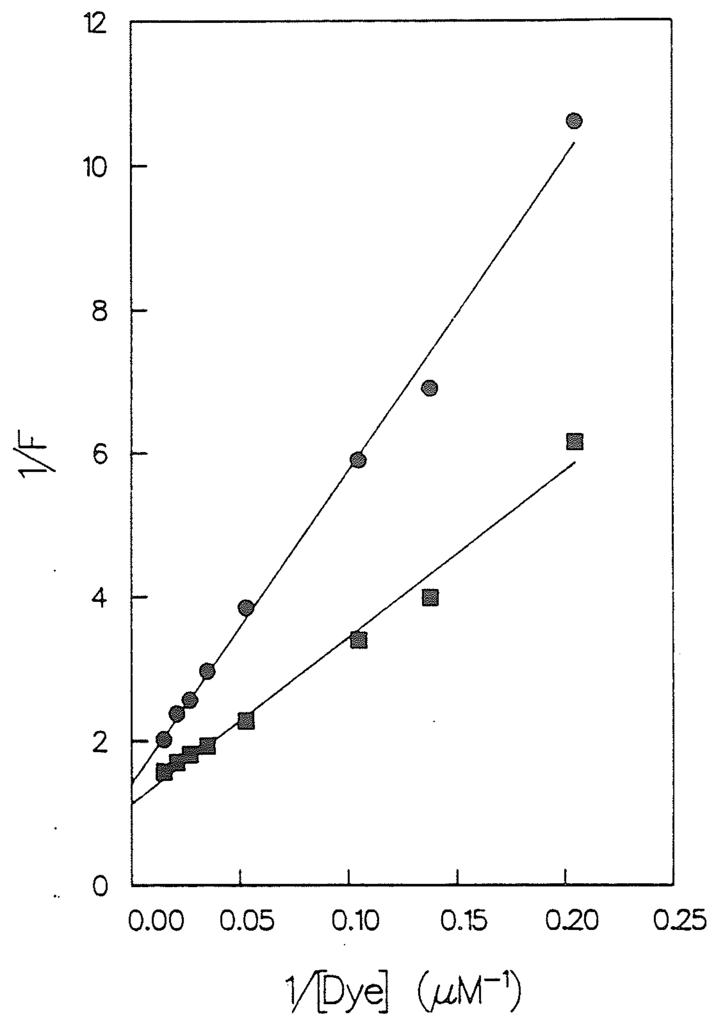


Figure 11. Double-reciprocal plots of fluorescence (F) quenching data for erythrosin (●) and rose bengal (■).

Protection From Photoinactivation by Edrophonium

Edrophonium, a competitive inhibitor of AChE (55), protected AChE from erythrosin-sensitized photoinactivation over a concentration range similar to that over which inhibition occurs (Figure 12). This protection occurred under both aerobic (data not shown) and anaerobic conditions. The non-competitive inhibitor d-tubocurarine, which binds to peripheral sites on the enzyme (56-58), provided no such protection at concentrations up to 1.0 mM (data not shown). Edrophonium could be protecting either of the sensitive residues tryptophan or histidine, both of which are known to be located at or near the substrate binding site of the enzyme (59,60).

The pH Dependence of Photoinactivation

The pH dependence of the rate of erythrosin-sensitized photoinactivation of AChE under both aerobic and anaerobic conditions is shown in Figure 13. The rate constants of both the aerobic and anaerobic reactions increase with increasing pH over the pH range 6.5 - 9.5, and this increase is much more dramatic under anaerobic conditions. The rates of both reactions increase dramatically at lower pH values (data not shown; see ref. 11). As we reported previously (11) the pH-dependent rate increase is much more dramatic under anaerobic conditions. The results of these more recent and detailed studies suggest that deprotonation of a sensitive residue increases the susceptibility of AChE towards erythrosin-sensitized photoinactivation. The residue in question appears to have a pK_a of approximately 6.6 (based upon

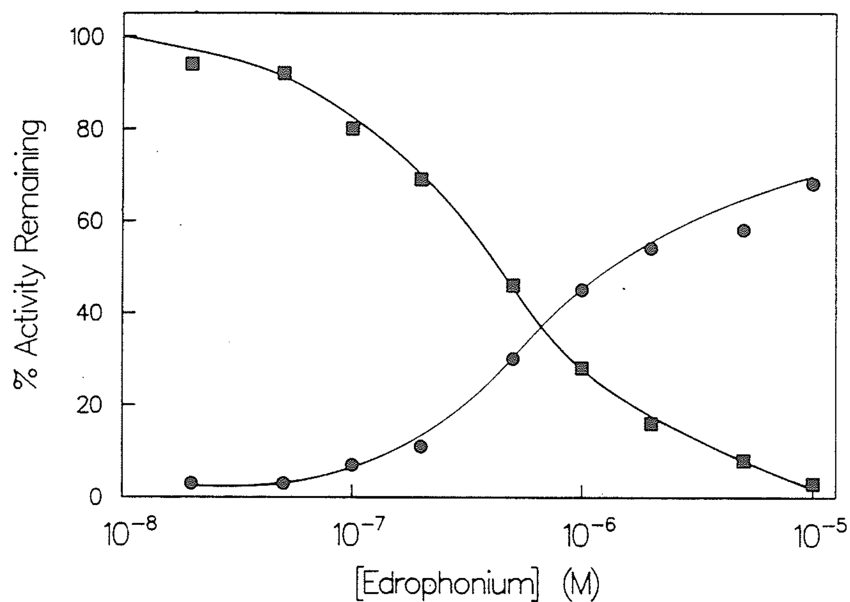


Figure 12. Protection of AChE from erythrosin-sensitized photoinactivation by edrophonium under anaerobic conditions. (■) Reversible inhibition of AChE by edrophonium alone. (●) Activity remaining after 10 min irradiation in the presence of edrophonium and 10 μM erythrosin.

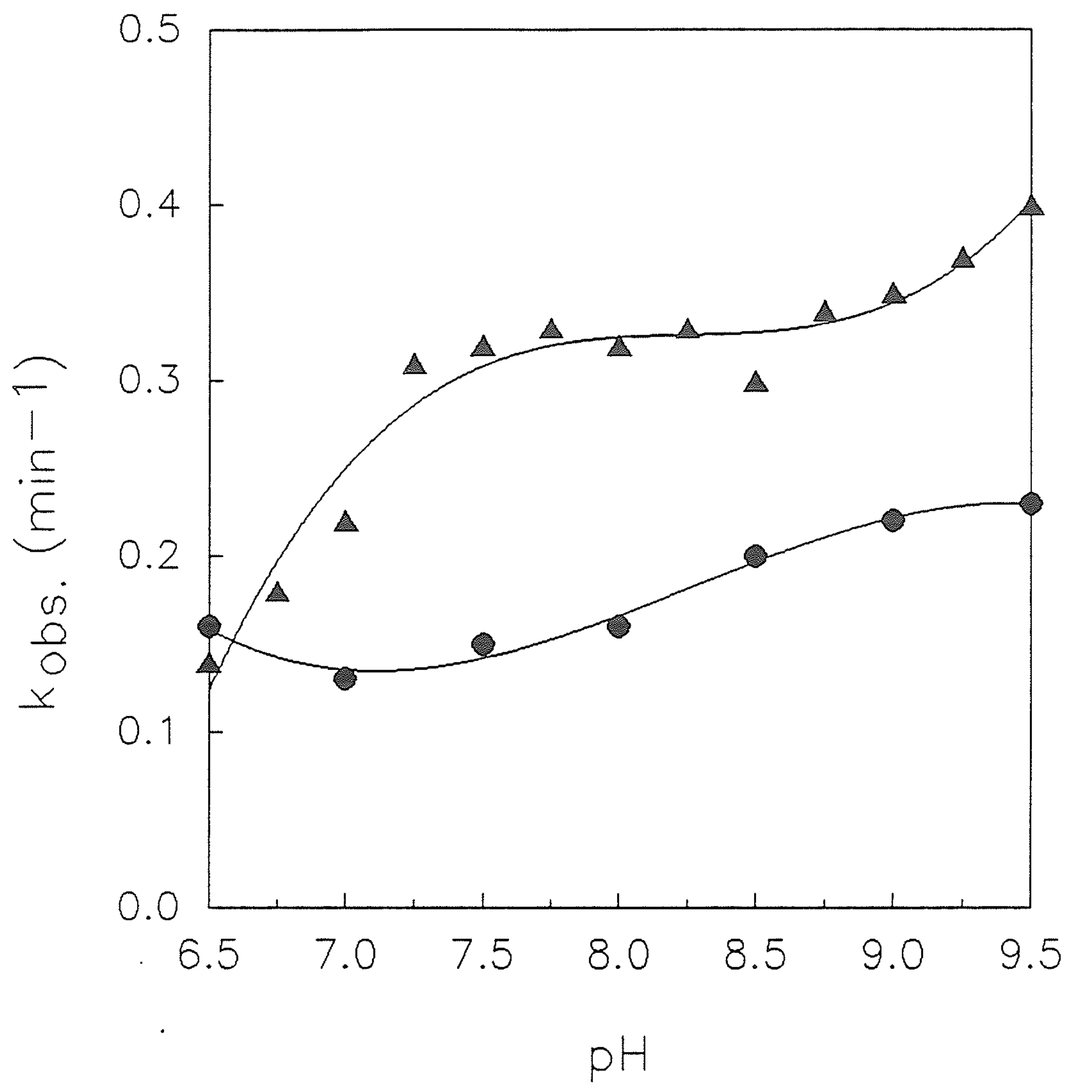


Figure 13. The effect of pH on the observed first-order rate constant of erythrosin-sensitized photoinactivation of AChE under aerobic (●) and anaerobic (▲) conditions.

extrapolation of the anaerobic pH-rate profile to $k_{obs.} = 0.0$).

Of the residues susceptible to dye-sensitized photooxidation only histidine has an ionizable side-chain with a pK_a close to 6.6. Histidine is further implicated as the residue responsible for this apparent titration curve by the fact that only the deprotonated form of histidine is susceptible (10). Finally, histidine is known to be involved in AChE catalysis (60,61). Destruction of this critical residue would certainly result in inactivation of the enzyme.

Amino Acid Analyses

In the early stages of this study it was found that amino acid analyses were not helpful in establishing the identity of the residue or residues involved in the erythrosin-sensitized photoinactivation of AChE. Technical problems with the analyzer and the fact that only a limited number of samples were examined resulted in data of very low precision. Meaningful comparisons of control and photoinactivated samples were therefore not possible. Subsequent analyses were carried out by the Protein Sequencing Facility at the University of Calgary and a much larger number of samples were examined.

The amino acid composition of untreated AChE is shown in Table 4. The results compare very favorably with previously published data and confirm that the enzyme preparation used in these studies was of very high purity.

Table 5 shows the amino acid compositions of untreated AChE subunits (as in Table 4) as well as those of anaerobically photoinactivated (10 μM erythrosin; >80% activity destroyed) AChE subunits at pH

Residue[1,2]	Residues per 70 kD subunit			
		Reference		
		(62)	(63)	(64)
Asp	75	65	78	72
Thr	29	26	27	24
Ser	45	41	40	40
Glu	66	56	61	66
Pro	44	48	35	41
Gly	54	46	52	53
Ala	36	33	37	44
Val	41	41	42	41
Met	16	18	16	--
Ile	24	22	22	24
Leu	58	53	51	49
Tyr	23	23	22	17
Phe	31	32	31	30
His	12	13	14	13
Lys	24	26	27	28
Trp	11	12	12	--
Arg	35	32	31	30

Table 4. Amino acid compositions of 70 kD subunits of 11s AChE from *Electrophorus electricus*.

[1] Cysteine was not measured (Cys is analyzed as cysteic acid which is a probable product of photooxidation).

[2] All values in this table were derived from 24 hr hydrolyses.

Table 5. Amino acid compositions of untreated and anaerobically photoinactivated 70 kD subunits of 11s AChE from *Electrophorus electricus*.

[1,2,3] As in Table 4.

[4] Results of triplicate analyses of photoinactivated samples (pH 8.0) which have standard deviations >1.0.

[5] Same as [4] but at pH 5.5.

[6] Glycine can be produced from tryptophan under some conditions.

[7] One set of triplicate analyses (His = 12) had a standard deviation of 0.44. The other set (His = 15) had a standard deviation of 1.32.

[8] One set of triplicate analyses had Trp = 0, one had Trp = 7.

Residue[1,2]	Residues per 70 kD subunit		
	control[3]	pH	
		5.5	8.0
Asp[4,5]	75	67	78
Thr[5]	29	35	31
Ser[5]	45	69	52
Glu	66	73	67
Pro[5]	44	36	41
Gly[4,5,6]	54	95	63
Ala[5]	36	40	37
Val[5]	41	28	40
Met	16	11	18
Ile	24	24	24
Leu[5]	58	47	56
Tyr[5]	23	24	23
Phe[4]	31	33	32
His[5,7]	12	13	9
Lys[5]	24	28	25
Trp	11	0	4[8]
Arg[5]	35	35	35

5.5 and pH 8.0. Several conclusions drawn from these and other results are summarized below.

i) The dramatic photoinactivation rate increases observed at low pH values which we reported previously (11) are due to increasing non-specificity in the photoinactivation mechanism, as indicated by the destruction of a wider variety of residues. However, Arg, Phe, Tyr and Ile seem to be resistant to erythrosin-sensitized photooxidation at all pH values.

ii) Destruction of histidine is responsible for the apparent titration curve observed under anaerobic conditions (see previous section and Figure 13).

iii) Erythrosin-sensitized photoinactivation of AChE does not involve the destruction of tyrosine under the conditions examined. This is also the case under aerobic conditions and in D_2O-based buffer (results not shown).

iv) Destruction of methionine may be involved in the photoinactivation process at lower pH values.

v) Protection of the enzyme by edrophonium does not seem to involve prevention of the loss of histidine under aerobic conditions but this could be a factor under anaerobic conditions (data not shown).

The inherent error involved in the amino acid analyses makes the detection of losses of small proportions of a particular residue rather difficult. Also, the experimental design contains the possibly invalid assumption that the photoinactivated enzyme is stable during gel filtration (erythrosin removal), drying, shipment and hydrolysis. Lower values of amino acids in some samples could be the result of

destruction during drying or hydrolysis. Increases in the amounts of amino acids could be due to at least two possible causes. Glycine can be produced from tryptophan under certain conditions (53) and our results suggest that this may be occurring in our samples (see Table 5) although the apparent relationship is by no means stoichiometric. Alternatively, the modification of an amino acid could result in the alteration of its elution position thereby causing it to be detected as a different amino acid (53). Photomodification of methionine may be impossible to detect since hydrolysis of the protein may also convert the photooxidation product of methionine to methionine (6).

CONCLUSION

The effectiveness of xanthenes as sensitizers of the photoinactivation of AChE parallels the triplet state quantum yields of the dyes studied. The quantum yields of singlet oxygen production also parallel the triplet state quantum yields but this does not aid in distinguishing between Type I and Type II reaction pathways.

The erythrosin-sensitized photoinactivation of AChE follows first-order kinetics at dye concentrations of 50 μM or lower. The hyperbolic nature of the dependence of the observed first-order rate constants of photoinactivation on erythrosin concentration under both aerobic and anaerobic conditions suggests that formation of an erythrosin-AChE complex may be involved in the photosensitization process. Alterations in the absorption spectrum of erythrosin in the presence of AChE as well as the observed quenching of AChE fluorescence by this dye both support this hypothesis.

Mechanistic studies involving the use of azide, a deuterated buffer and oxygen removal by argon gassing indicate that under aerobic conditions the erythrosin-sensitized photoinactivation of AChE proceeds by a mixed Type I - Type II reaction mechanism. In H_2O-based buffer the incomplete quenching of sensitized photoinactivation as well as the elevated rate under anaerobic conditions where azide has little or no effect suggests that the Type I mechanism proceeds more quickly in the absence of oxygen than does the mixed Type I - Type II in the presence of oxygen. Tests involving D_2O-based buffer support these conclusions and also indicate that the Type I mechanism may be affected by this solvent.

Edrophonium protects AChE from erythrosin-sensitized photoinactivation at concentrations which imply that binding of edrophonium at the catalytic site of the enzyme affords this protection. Additionally, the pH dependence of the rate of photoinactivation under anaerobic conditions as well as the known proximity of histidine to the catalytic site provide compelling evidence for the conclusion that the destruction of histidine is involved in the photoinactivation process. This is further supported by the results of amino acid analyses which indicate that erythrosin-sensitized destruction of histidine is pH-dependent. It seems reasonable to speculate that erythrosin might bind to AChE at or near this catalytically-important histidine residue under both aerobic and anaerobic conditions. The bound dye could then react directly with histidine *via* a Type I mechanism. Alternatively, singlet oxygen could be generated by the bound dye and react with histidine as well as other sensitive residues thus causing photoinactivation by a Type II mechanism as well as reducing the significance of the Type I pathway. Either of these reaction mechanisms would lead to the relatively specific destruction of a few residues. Given the anionic nature of AChE (pI = 5.0, ref. 54, 65) and the hydrophobic nature of erythrosin, the marked increase in the rates of photoinactivation at lower pH values under both aerobic and anaerobic conditions could be due to an increase in non-specific binding of the dye to the enzyme surface as the enzyme approaches its isoelectric point. This would lead to much more rapid photoinactivation of the enzyme.

Amino acid analyses aimed at conclusively establishing the nature of the susceptible residue or residues involved in the erythrosin-sensitized photoinactivation of AChE have been fruitful but not defin-

itively conclusive. Tyrosine is not involved in the photoinactivation process under any of the conditions examined. Tryptophan destruction is extensive or complete under the conditions studied but loss of this residue may or may not be directly related to the loss of enzyme activity. The observed pH-dependence of histidine destruction implicates this residue as that which confers sensitivity upon AChE towards the apparently more specific mechanism of erythrosin-sensitized photoinactivation at pH values above 6.5 under anaerobic conditions. The protection of the enzyme by edrophonium under these conditions supports this conclusion. The relationship between loss of AChE activity and histidine destruction has never been demonstrated by direct methods prior to this report.

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