

AD-R175 358

SYNTHESIS OF A PHOTSENSITIVE
DI-(P-AZIDOPHENYL)CHLOROPHOSPHATE AND THE K. (U)
CHEMICAL RESEARCH AND DEVELOPMENT CENTER ABERDEEN
PROVING GRO . . H R YEH OCT 86

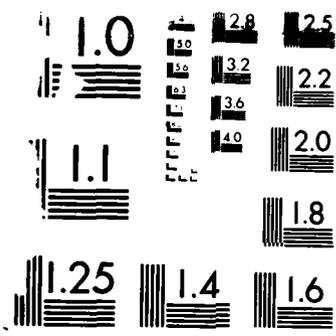
1/1

UNCLASSIFIED

F/G 15/2

NL





1951-1952 RESOLUTION TEST CHART
NBS 1951-1952

12

**CHEMICAL
RESEARCH,
DEVELOPMENT &
ENGINEERING
CENTER**

CRDEC-TR-87007

**SYNTHESIS OF A PHOTSENSITIVE
DI-(p-AZIDOPHENYL)CHLOROPHOSPHATE
AND THE KINETICS OF THE LIGHT-DEPENDENT
INHIBITION OF HORSE SERUM
CHOLINESTERASE**

AD-A175 358

DTIC FILE COPY

by **Homer R. Yeh, Ph. D.**
RESEARCH DIRECTORATE

October 1986

DTIC
S E D
DEC 29 1986
E

U.S. ARMY
ARMAMENT
MUNITIONS
CHEMICAL COMMAND



Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

Distribution Statement

Approved for public release; distribution is unlimited.

AD-A175357

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED		1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY		3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.	
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE			
4. PERFORMING ORGANIZATION REPORT NUMBER(S) CRDEC-TR-87007		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION CRDEC	6b. OFFICE SYMBOL (if applicable) SMCCR-RSB	7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423		7b. ADDRESS (City, State, and ZIP Code)	
8a. NAME OF FUNDING / SPONSORING ORGANIZATION CRDEC	8b. OFFICE SYMBOL (if applicable) SMCCR-RSB	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423		10. SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO. 1L161102	TASK NO. A71A
		PROJECT NO.	WORK UNIT ACCESSION NO.
11. TITLE (Include Security Classification) Synthesis of a Photosensitive Di-(p-Azidophenyl)chlorophosphate and the Kinetics of the Light-Dependent Inhibition of Horse Serum Cholinesterase			
12. PERSONAL AUTHOR(S) Yeh, Homer R., Ph.D.			
13a. TYPE OF REPORT Technical	13b. TIME COVERED FROM 85 Apr TO 85 Jun	14. DATE OF REPORT (Year, Month, Day) 1986 October	15. PAGE COUNT 21
16. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD 15	GROUP 02	Di-(p-azidophenyl)chlorophosphate Kinetics Horse serum cholinesterase Photosensitive Light-dependent inhibition	
19. ABSTRACT (Continue on reverse if necessary and identify by block number) <i>has been</i> Aromatic azides are reasonably stable and can be easily synthesized without drastic reaction conditions. The most popular method was reported by P.A.S. Smith. Following Smith's method, I have prepared an azido derivative, namely: di-(p-azidophenyl)chlorophosphate (DAPP). The presence of azido groups in the molecule enables its direct linkage with solid supports and proteins by a simple photolysis procedure. The newly synthesized compound has strong ultraviolet absorption at 276 nm and the characteristic azide band at 2100/cm ⁻¹ . Unlike diisopropylfluorophosphate (DFP), neither DAPP nor its irradiated product had any inhibitory effect on horse serum cholinesterase. However, simultaneous presence of DAPP and the enzyme in the reaction mixtures under UV irradiation resulted in strong inhibition.			
(Continued on reverse)			
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED	
22a. NAME OF RESPONSIBLE INDIVIDUAL TIMOTHY E. HAMPTON		22b. TELEPHONE (Include Area Code) (301) 671-2914	22c. OFFICE SYMBOL SMCCR-SPS-T

1

.000001

.00001

of the enzyme activity. The light-dependent DAPP inhibition of cholinesterase depended on DAPP concentrations present. The concentration that yielded half-maximal velocity was in the range of 1.0×10^{-7} M and the presence of 10^{-5} M DAPP almost completely inhibited the enzyme reaction. The light-dependent DAPP inhibition of cholinesterase resulted in the reduction of both K_m and V_{max} values of the enzyme system. Since the reciprocal plots of the kinetic data did not intersect at a common point, and also because the slopes vs concentration replots were nonlinear, the presence of two or more nonmutually exclusive inhibitor binding sites is predicted.

(K_m vs [DAPP] replot)

PREFACE

The work described in this report was authorized under Project No. 1L161102A71A, Research in Chemical & Biological Defense, Biotechnology. This work was started in April 1985 and completed in June 1985. The experimental data are recorded in laboratory notebook 85-0109.

The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

Reproduction of this document in whole or in part is prohibited except with permission of the Commander, U.S. Army Chemical Research, Development and Engineering Center, ATTN: SMCRR-SPS-T, Aberdeen Proving Ground, Maryland 21010-5423. However, the Defense Technical Information Center and the National Technical Information Service are authorized to reproduce the document for U.S. Government purposes.

This report has been approved for release to the public.

Acknowledgment

The author wishes to thank Cecilia B. Doughty for her careful editing of this report.

Accession For	
NTIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input checked="" type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

Blank

CONTENTS

1.	INTRODUCTION	7
2.	MATERIALS AND METHODS	7
2.1	Chemicals	7
2.2	Photolysis	8
2.3	Enzyme Kinetics.	8
3.	RESULTS	8
3.1	Identification of the Chemical Structure of DAPP	8
3.2	Kinetics of DAPP Inhibition of Cholinesterase.	10
4.	DISCUSSION	15
	LITERATURE CITED	21

Blank

SYNTHESIS OF A PHOTSENSITIVE DI-(p-AZIDOPHENYL)CHLOROPHOSPHATE AND THE KINETICS OF THE LIGHT-DEPENDENT INHIBITION OF HORSE SERUM CHOLINESTERASE

1. INTRODUCTION

Diisopropylfluorophosphate (DFP), an organophosphate, has been extensively used for affinity labeling of the serine-containing esterases, such as cholinesterases. There are well over 20 serine residues in these enzyme molecules, but only the one at the active center is reactive.¹⁻⁵ Since some of the anticholinesterases may have inhibitory constant (K_i) in the order of 10^{-9} li,⁶ they are too toxic for routine laboratory use in investigation of these physiologically important enzymes and other related neuroreceptors. Therefore, one of the major goals of this study was to search for analogs of less toxicity. The final results may have other therapeutic and toxicological significance.

Aromatic azides are reasonably stable and can be easily synthesized without drastic reaction conditions. Upon irradiation, both the photochemically generated singlet and triplet nitrene-free radicals are highly reactive and show electrophilic character.⁷⁻⁹ Singlet nitrene is especially reactive with hydroxyl amino groups by insertion into oxygen-hydrogen or nitrogen-hydrogen bonds. Thus, it appears that aryl azides are ideal reagents for photoaffinity labeling in the study of the structural and functional relationships of these enzymes and neuroreceptors.

As a result of our present efforts, an azido derivative, namely: di-(p-azidophenyl)chlorophosphate (DAPP) was synthesized in our laboratory and obtained in pure crystalline form. The results of our investigation on the synthesis of this photosensitive compound and its kinetics of the light-dependent inhibition of horse serum cholinesterase are reported here.

2. MATERIALS AND METHODS

2.1 Chemicals.

p-Aminophenol was obtained from the IICB Chemical Co., Inc., Cincinnati, OH. Triethylamine and phosphorus oxychloride were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Acetylthiocholine (ATCH), dithiobisnitrobenzoate (DTNB), diisopropylfluorophosphate (DFP), and horse serum cholinesterase were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were reagent grade or the highest purity and were used without further purification.

p-Azidophenol and p-azidophenylphosphate were prepared from p-aminophenol according to the methods described by Forster and Fierz.^{10,11} Di-azidophenyl)chlorophosphate was synthesized in our laboratory according to the following procedure. To a closed flask protected from light, fitted with a magnetic stirrer, and containing a solution of triethylamine (2.0 ml) in 100 ml of dry ether cooled to -5°C , 500 mg of p-azidophenol was added, followed by the addition of 0.19 ml of phosphorus oxychloride. The mixture was stirred continuously in the cold for 3-4 hours and then allowed to warm up gradually to

room temperature while stirring continued overnight. After the mixture was evaporated to dryness, the residue was dissolved in 200 ml of dry ether and filtered to remove any insoluble substances. The clear ether solution was washed twice with 100 ml of 0.1 N of sodium hydroxide and then washed several times with a sufficient amount of deionized water to remove alkali and any unreacted phenolic compounds. After drying the ether layer over anhydrous sodium sulfate, the solvent was removed under reduced pressure to give about 400 mg of the crude product. This was then recrystallized from peroxide-free dry ether and yielded about 300 mg of light-brown crystals of a melting point of 89.4 °C. Yield: 40 percent.

2.2 Photolysis.

Photolysis was conducted with a short UV lamp, UVS-11 (Ultra-Violet Products, Inc., San Gabriel, CA). All enzyme solutions, in the presence or absence of the inhibitor, were irradiated in the cold at 4 °C for 3 minutes in a quartz cuvette of 1-cm pathlength, kept 2 cm from the window of the UV lamp. Immediately following this photolysis procedure, cholinesterase activity was determined by mixing the irradiated enzyme solutions with the substrate solutions.

2.3 Enzyme Kinetics.

For studies of the DAPP concentration effect, the irradiated enzyme solutions contained 3 µg of cholinesterase and from 1.78×10^{-10} to 1.78×10^{-6} M of DAPP in 1.06 ml of 0.1 M of sodium phosphate buffer, pH 7.4. The substrate solutions consisted of 1.0×10^{-3} M of ATCH in 1.0 ml of the phosphate buffer. DAPP was added in 20 µl ethanol, and the same amount of the solvent was also added to all controls to compensate for the solvent effect. After mixing the enzyme and the substrate solutions, the final DAPP concentrations of the reaction mixtures varied from 9.0×10^{-11} to 9.0×10^{-7} M, while the substrate concentration was fixed at 4.8×10^{-4} M. To investigate the effect of substrate concentrations, the reaction mixtures contained 50 µg the enzyme, 1.1×10^{-8} M, 1.1×10^{-7} M, and 1.1×10^{-6} M fixed levels of DAPP, and substrate varied from 4.8×10^{-7} to 3.6×10^{-6} M, in a total volume of 2.1 ml per cuvette. Immediately after mixing of the substrate (1.08 ml) and the enzyme (1.0 ml) solutions, cholinesterase activity was monitored by the addition of 20 µl of 5.9×10^{-2} M of thiol reagent DTNB in phosphate buffer, and the rate of formation of the yellow product was followed at 420 nm in a Cary 210 spectrophotometer maintained at 25 °C.

3. RESULTS

3.1 Identification of the Chemical Structure of DAPP.

Silica gel thin-layer chromatography (TLC), developed in chloroform, gave a single spot for both the starting material, p-azidophenol (AP), and the product, DAPP. R_f of AP = 0.19; R_f of DAPP = 0.60. Analysis: MS: m/e 351; UV: $\lambda_{\text{Max}}^{\text{EtOH}}$ 250 (molar extinction coefficient 3.46×10^4). IR: $\lambda_{\text{Max}}^{\text{KBr}}$ 4.76, 6.65, 7.70, 8.34, 8.55, 10.25, and 11.8 µm. The broad phenolic peaks shown by AP at 2.95 and 8.0 µm were not observed for DAPP. The IR spectra of AP and DAPP are shown in Figure 1. The chemical structure of DAPP was further confirmed by elementary analysis and NMR spectroscopy.

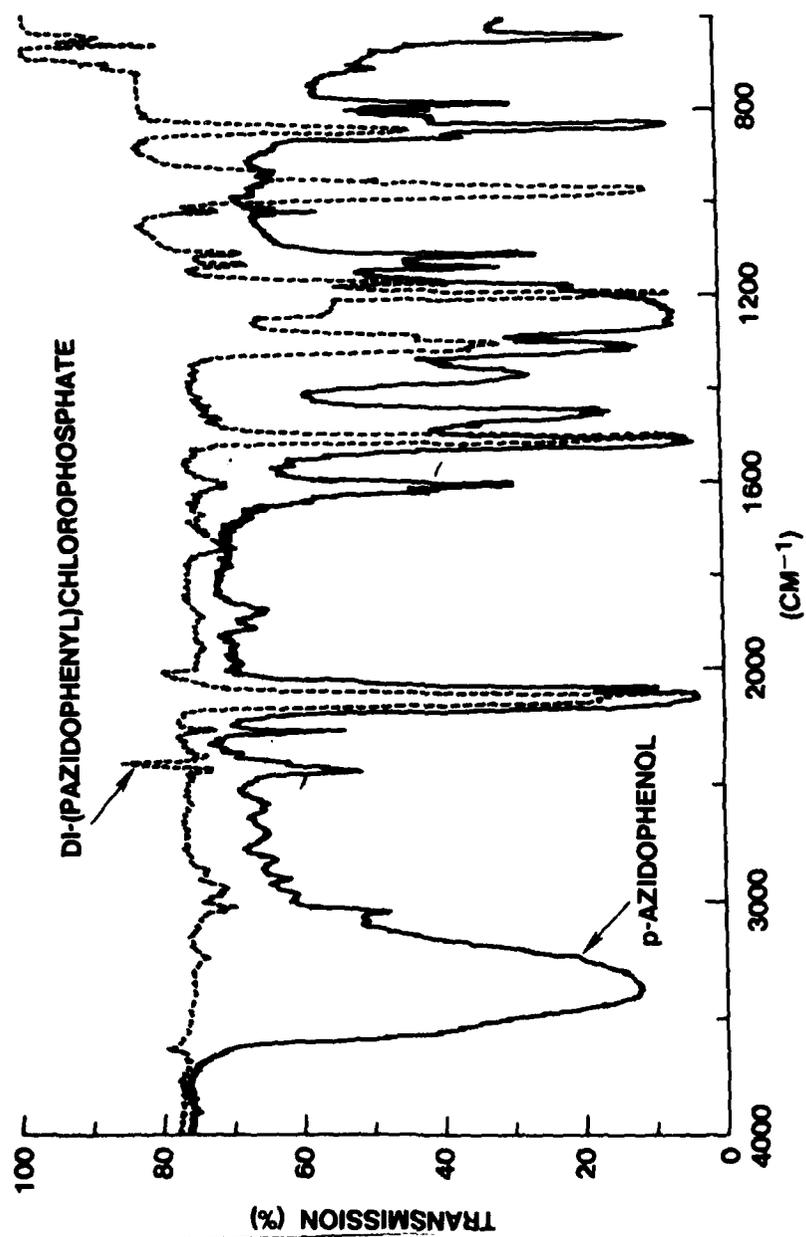


Figure 1. Infrared Absorption Spectra of p-Azidophenol and di-(p-Azidophenyl)chlorophosphate in KBr Pellet

3.2 Kinetics of DAPP Inhibition of Cholinesterase.

3.2.1 Effect of DAPP Photolysis on Cholinesterase Activity.

The effect of UV light and DAPP on the rate of ATCH hydrolysis by cholinesterase is shown in Figure 2. Curves 1, 2, and 3 represent the cholinesterase activity of the control experiments obtained: (a) by irradiation of the enzyme in the absence of DAPP; (b) with added DAPP but in the absence of irradiation; and (c) by mixing irradiated DAPP with cholinesterase that was not irradiated. Curve 4 of Figure 4 was obtained by irradiation of the enzyme in the presence of DAPP. These results clearly indicate that neither DAPP nor its irradiated products had any significant effect on the activity of the enzyme system. However, when irradiation took place with the simultaneous presence of the enzyme and DAPP in the reaction mixtures, the enzyme activity was strongly inhibited (Curve 4). These observations suggest that DAPP inhibition of the cholinesterase was light-dependent and the light-generated reactive groups had extremely transient half-lives, which was consistent with those chemical characteristics of nitrene-free radicals.

3.2.2 Effect of DAPP Concentrations on the Light-Dependent Inhibition of Cholinesterase.

The effect of DAPP concentrations on the light-dependent inhibition of horse serum cholinesterase is shown in Figures 3 and 4. Figure 3 shows the velocity curves of cholinesterase hydrolysis of the substrate (ATCH) in the presence and absence of varying amounts of DAPP. The results indicate that the inhibition of ATCH hydrolysis by DAPP increases with increasing concentrations of DAPP. The plot of the percentage of inhibition vs DAPP concentrations gave a linear curve (Figure 4). Under the reaction conditions employed, the concentration required to reduce the reaction to half its maximal velocity was in the range of 9.0×10^{-10} M. The presence of 9.0×10^{-7} M of DAPP almost completely inhibited the enzymatic hydrolysis of the substrate.

3.2.3 Effect of DAPP on K_m and V_{max} of the Light-Dependent Inhibition of Cholinesterase.

The effect of DAPP on K_m and V_{max} values of the enzyme reaction is shown in Figure 5. The Lineweaver-Burk plots of the kinetic data were obtained in the presence and absence of irradiation. Curves 1 and 2 were control curves. Curves 3, 4, and 5 were obtained in the presence of 1.1×10^{-8} M, 1.1×10^{-7} M, and 1.1×10^{-6} M of DAPP, respectively. The K_m values, calculated by the Teast-square method, were found to be 3.0×10^{-8} M, 2.2×10^{-8} M, and 1.3×10^{-8} M, respectively. The corresponding V_{max} values were estimated to be 0.87, 0.56, and 0.29 absorbance/minute, respectively. The light-dependent DAPP inhibition of the enzyme reaction resulted in reduction of both the K_m and V_{max} values of the system. The results may indicate either an uncompetitive or a mixed type inhibitory mechanism. The replots of the slopes vs [I], or intercepts vs [I], yielded a nonlinear curve, indicating a mixed type inhibitory mechanism. Thus, K_i , DAPP was calculated from secondary plots of $1/\Delta$ slope vs $1/[DAPP]$ and $1/\Delta$ intercept vs [DAPP]. The secondary replots gave linear lines. Accordingly, K_i , DAPP was estimated to be in the range of 1.3×10^{-7} M. The K_i value does not necessarily reflect the concentration of DAPP required to reduce the reaction rate to half of its maximal value. The concentrations of DAPP required to reduce the reaction to half of its maximal velocity varied with the

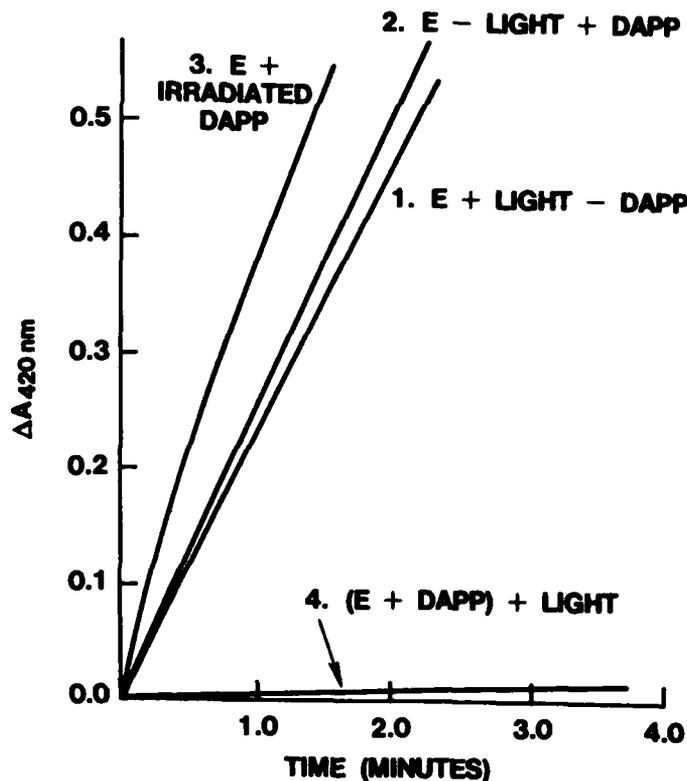


Figure 2. Effect of DAPP Photolysis on the Activity of Horse Serum Cholinesterase (ChE)

Photolysis was carried out in a mixture containing 3 μg of ChE in 1.06 ml of 1.0 M of Na_2PO_4 buffer, pH 7.4, in the presence or absence of 0.02 ml of 9.5×10^{-5} M of DAPP. Enzyme reaction was initiated by mixing irradiated solutions with substrate solutions that contained 1.0 ml of 10^{-3} M of ACTH and 0.02 ml of 1.2×10^{-2} M of DTNB. The final reaction mixture contained 3 μg of ChE, 4.8×10^{-4} M of ACTH, and 5.6×10^{-4} M of DTNB, in the presence or absence of DAPP. Total volume of the reaction mixture was 2.1 ml. Enzyme activity was monitored at 420 nm in a Cary 210 spectrophotometer.

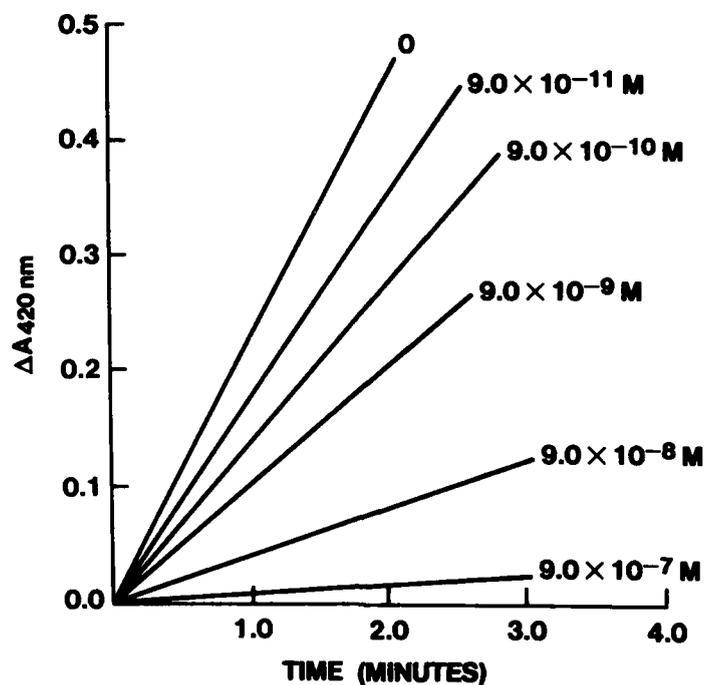


Figure 3. Effect of DAPP Concentrations on the Light-Dependent Inhibition of Cholinesterase

Photolysis was carried out in a reaction mixture that contained 50 μ g of cholinesterase in 1.06 ml of 1.0 M of sodium phosphate buffer, pH 7.4, in the presence or absence of DAPP. Concentrations of DAPP were fixed at levels of 2.3×10^{-8} M (Curve 3), 2.3×10^{-7} M (Curve 4), and 2.3×10^{-6} M (Curve 5). Enzyme reaction was initiated by mixing of the substrate and the enzyme solutions. The final reaction mixtures contained 1.1×10^{-8} M, 1.1×10^{-7} M, and 1.1×10^{-6} M, respectively, of the fixed levels of DAPP, 5.6×10^{-4} M of DTNB, and from 4.8×10^{-7} to 3.6×10^{-7} M of ATCH. Curve 1 was a control obtained in the absence of both irradiation and DAPP. Curve 2 was a control obtained in the presence of irradiation, but in the absence of DAPP. Total volume of the reaction mixture was 2.1 ml per cuvette. Enzyme activity was monitored at 420 nm in a Cary 210 spectrophotometer.

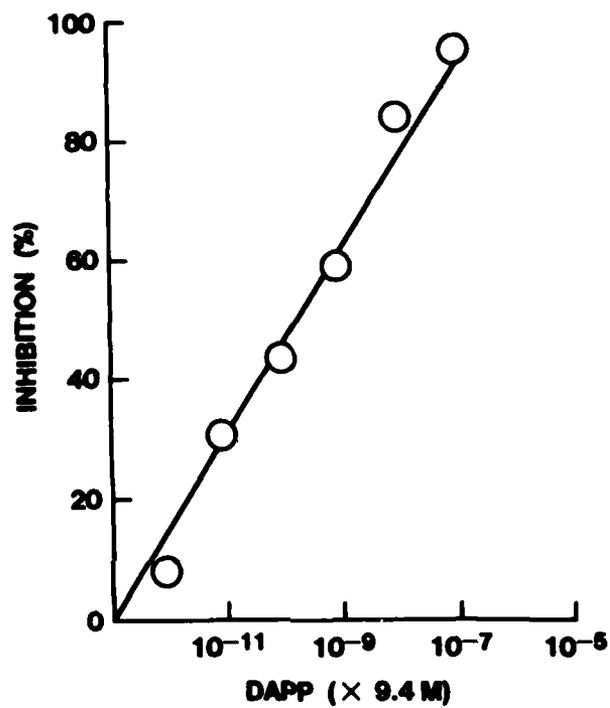


Figure 4. Percentage of Inhibition vs DAPP Concentration Plot
Data were obtained from Figure 3.

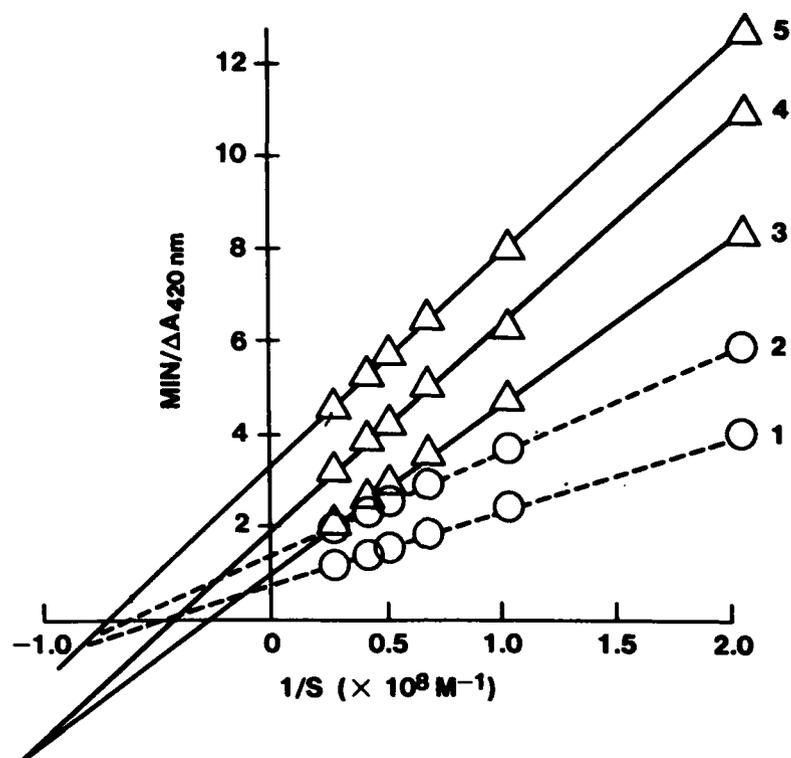


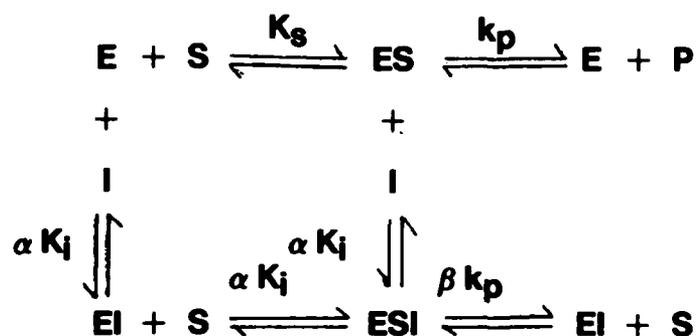
Figure 5. Effect of DAPP Concentrations on K_m and V_{max} Values of ATCH Hydrolysis by Cholinesterase

Photolysis was carried out in a reaction mixture that contained 50 μg of cholinesterase in 1.06 ml of 1.0 M of sodium phosphate buffer, pH 7.4, in the presence or absence of DAPP. Concentrations of DAPP were fixed at levels of 2.3×10^{-8} M (Curve 3), 2.3×10^{-7} M (Curve 4), and 2.3×10^{-6} M (Curve 5). Enzyme reaction was initiated by mixing of the substrate and the enzyme solutions. The final reaction mixtures contained 1.1×10^{-8} M, 1.1×10^{-7} M, and 1.1×10^{-6} M, respectively, of the fixed levels of DAPP, 5.6×10^{-4} M of DTNB, and from 4.8×10^{-7} to 3.6×10^{-7} M of ATCH. Curve 1 was a control obtained in the absence of both irradiation and DAPP. Curve 2 was a control obtained in the presence of irradiation, but in the absence of DAPP. Total volume of the reaction mixtures was 2.1 ml per cuvette. Enzyme activity was monitored at 420 nm in a Cary 210 spectrophotometer.

enzyme concentrations present. At this point, it may be worthwhile to note that, as the fixed inhibitor concentration was increased, all the plus inhibitor and control plots did not intersect at a common point. The effects of DAPP photolysis on the K_m and V_{max} values of the enzyme systems are listed in Table 1.

4. DISCUSSION

The general reaction scheme for the equilibrium type inhibition is shown below.



The mechanism of the mixed type reaction depends greatly on the relative values of α and β of the equilibrium scheme.¹² Since the replots of the slopes vs [DAPP] and intercepts vs [DAPP] both gave nonlinear curves, the relative values of α and β can not be estimated directly from these replots. These values were then calculated from the secondary plots of $1/\Delta$ slope vs $1/[\text{DAPP}]$ and $1/\Delta$ intercept vs $1/[\text{DAPP}]$. Accordingly, α and β values were found to be in the range of 1.4 and 0.34, respectively. The relative magnitude of α and β suggests that both free cholinesterase and E-DAPP can bind with the substrate, but with different affinities. And both ES and E-DAPP-S can form product, but at different rates. If this is correct, the inhibition of cholinesterase by photolysis of DAPP may result from a mixture of partial non-competitive and partial competitive inhibition of the enzyme. Thus the kinetic data may implicate the presence of more than one distinct inhibitor-binding site on the enzyme molecules. The binding of one site may affect the binding of the other; however, they are not mutually exclusive. This possibility may be supported by the observation that the double reciprocal plots of the plus inhibitor and control experiments did not intersect at a common point. Alternatively, the binding of the enzyme with DAPP may result in the change of the enzyme conformation, and more than one conformational form exists with slightly modified binding and catalytic behavior.

Two distinct kinds of affinity-labeling reagents, endo and exo, have been reported.¹³ Endo affinity-labeling reagents are those that covalently bind to amino acids within the active center. Exo reagents usually are substrate or inhibitor analogs having the ability to bind with both active or

Table 1. Effect of DAPP Photolysis on Kinetic Constants of ATCH Hydrolysis by Cholinesterase

<u>DAPP CONCENTRATION (M)</u>	<u>IRRADIATION (MINUTES)</u>	<u>K_m (× 10⁻⁸ M)</u>	<u>V_{max} (OD/MINUTE)</u>
NONE	NONE	1.9	1.26
NONE	3	1.5	0.67
1.1 × 10 ⁻⁸	3	3.0	0.87
1.1 × 10 ⁻⁷	3	2.2	0.56
1.1 × 10 ⁻⁶	3	1.3	0.29

receptor sites and secondary sites on the protein molecules. DFP is an exclusive endo affinity-labeling agent.¹⁴ However, unlike DFP, DAPP may be classified as an exo affinity-labeling agent, similar to 4-azido-(2-nitrobenzyl)triethylammonium¹⁵ and benzamidine sulfonyl fluorides.¹⁶ The effect of DAPP on neuroreceptors and the sodium and potassium pump of nerve fibers will be further investigated.

Under similar reaction conditions, DFP inhibition of the horse serum cholinesterase followed a partial competitive inhibition distinct from that observed for DAPP. The kinetic data of DFP inhibition of the cholinesterase is shown in Figure 6 and Table 2.

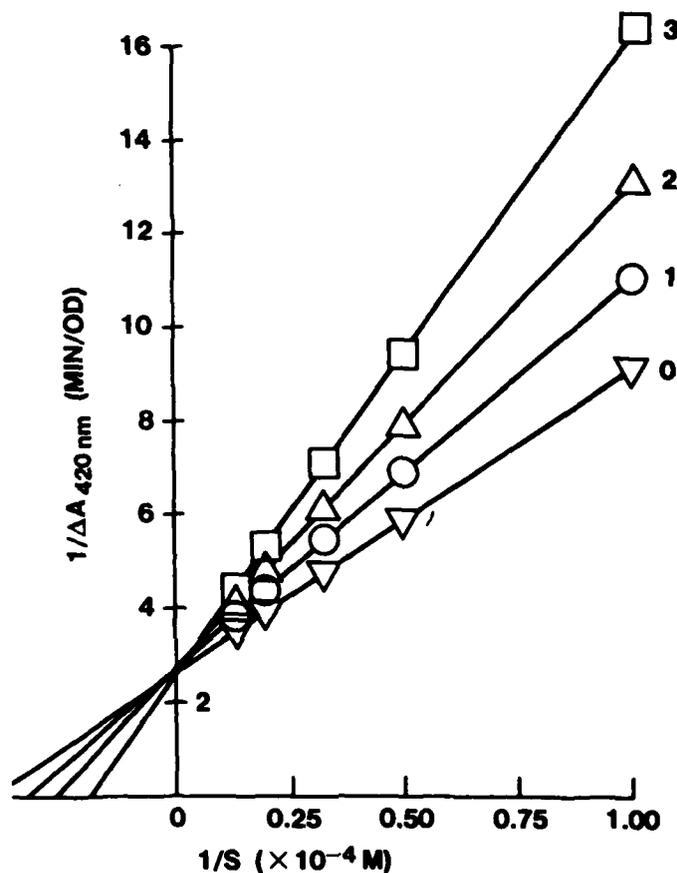


Figure 6. Effect of DFP Concentrations on K_m and V_{max} Values of Cholinesterase Activity

Reaction mixtures contained 0 (Curve 0), 9.3×10^{-8} M (Curve 1), 1.8×10^{-8} M (Curve 2), and 2.79×10^{-7} M (Curve 3) of the fixed levels of DFP. Substrate concentrations were varied from 1.0×10^{-4} M to 7.5×10^{-4} M. Reaction was initiated by the addition of 10 μ g of the enzyme in 20 μ l of the phosphate buffer. The velocity of the enzyme reaction was monitored by addition of 20 μ l of 5.6×10^{-4} M DTNB and followed at 420 nm in a Cary 210 spectrophotometer at 25 °C.

Table 2. Effect of DFP on K_m and V_{max} of the Cholinesterase Hydrolysis of ATCH

<u>DFP CONCENTRATION (M)</u>	<u>K_m ($\times 10^{-4}$ M)</u>	<u>V_{max} (OD/MINUTE)</u>
WITHOUT	2.42	0.373
9.3×10^{-8}	3.09	0.366
1.8×10^{-7}	3.79	0.358
2.8×10^{-7}	5.60	0.399

Blank

LITERATURE CITED

1. Schaffer, N.K., Simet, L., Harshman, S., Engle, R.R., and Drisko, R.W. *J. Biol. Chem.* 225, 147 (1957).
2. Turba, F., and Gundlach, G. *Biochem. J.* 327, 186 (1955).
3. Naughton, M.A., Sanger, F., Hartley, B.S., and Shaw, D.C. *Biochem. J.* 77, 149 (1960).
4. Gladner, J.A., and Laki, K. *J. Am. Chem. Soc.* 80, 1263 (1958).
5. Sanger, F., and Shaw, D.C. *Nature (London)* 187, 872 (1960).
6. Webb, E.C. *In Symposium on the Biochemical Reactions of Chemical Warfare Agents*, p 50. R. T. Williams, Ed. *Biochem. Soc. Symposium No. 2*. Cambridge University Press. 1948.
7. Smith, P.A.S., and Brown, B.B. *J. Am. Chem. Soc.* 73, 2435 (1951).
8. McRobbie, I.M., Meth-Cohn, O., and Suschitzky, H. *Tetrahedron Letter*. pp 925 and 929. (1976).
9. Bayley, H., and Knowles, J.R. *In Methods Enzymology*, pp 69-114. W. B. Jakoby, and M. Wilchek, Eds. *Academic Press*, New York, NY. 1977.
10. Forster, M.O., and Fierz, H.E. *J. Chem. Soc.* 91, 855 (1907).
11. Forster, M.O., and Fierz, H.E. *J. Chem. Soc.* 91, 1850 (1907).
12. Segel, I.H. *Ezyme Kinetics*. Chapter 4, pp 161-226. *John Wiley & Sons, Inc.*, New York, NY. 1975.
13. Baker, B.R. *J. Pharm. Sci.* 53, 347 (1964).
14. Janes, H.S., Posthumus, C.H., and Cohen, J.A. *Biochim. Biophys. Acta* 33, 387 and 396 (1959).
15. Hucho, F., Bergman, D., Dubois, J.M., Rojas, E., and Kiefer, H. *Nature* 260, 902 (1976).
16. Cory, M., Andrews, J.M., and Bing, D.H. *Methods Enzymology*. Vol. XLVI, p 129. 1977.

END

2-87.

DTIC