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FEASIBILITY OF MONITORING PESTICIDE BREAKTHROUGH FROM CHARCOAL COLUMNS

FINAL COMPREHENSIVE REPORT

by

Donald R. Sellers, Ph.D.

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Eel cholinesterase test tickets and procedures were tested and modified to be sensitive to Baygon®, carbaryl, diazinon, Dursban®, and malathion for the rapid detection of these pesticides in water. Detection limits for all the pesticides in water were below 10 ppm using the enzyme test tickets and pre-treatment with bromine. The tickets were stable during storage at 4°C or 40°C for at least 8 months. Bimolecular rate constants were determined for the inhibition of cholinesterase in solution.

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FOREWORD

The effort described in this final comprehensive report spanned the period from September 1, 1978 to October 30, 1979. More detailed discussions and additional supportive data may be obtained by consulting the 10 monthly technical progress reports covering this work. These reports may be obtained by writing to the COTR, Dr. C. W. R. Wade, U.S. Army Medical Bioengineering and Research Development Laboratory, Fort Detrick, Frederick, MD 21701.

The author wishes to acknowledge the technical advice of Dr. Louis H. Goodson, the managerial assistance of Mr. William B. Jacobs, and the technical expertise of Mr. Michael Kalinoski, Mrs. Julie Kelly, and Miss Valerie Mitts. We are grateful to Mr. Wally Sauer of the McKenna Company, St. Louis, for supplying us with sample packaging materials.

ABSTRACT

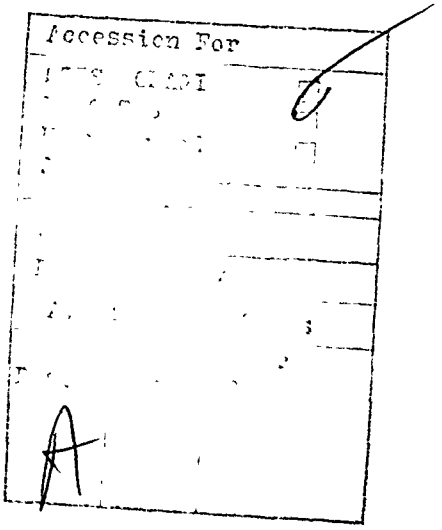
Eel cholinesterase test tickets and procedures developed under a previous Army contract (Contract No. DAAA15076-C-0132) were tested and modified to be sensitive to Baygon®, carbaryl, diazinon, Dursban®, and malathion for the rapid detection of these compounds in water at proposed Army pesticide filtration plants. Sensitivities to the phosphorothionates diazinon, Dursban®, and malathion were greatly enhanced by prior treatment with bromine water. Detection limits for all the pesticides in water were below 10 ppm. Interference with the enzyme test by solubilizing agents expected in pesticide formulations and metal ions at environmentally important concentrations was tested.

The enzyme test tickets were stable during storage at 4°C or 40°C for at least 8 months.

The scientific literature was searched using classical manual methods as well as computerized techniques for tests similar to the enzyme tickets for detection of chlorinated hydrocarbon pesticides in wastewater.

Bimolecular reaction rate constants were determined for the inhibition of cholinesterase in solution and compared with enzyme ticket detection limits.

Ten pesticide test kits were prepared and submitted to the sponsor. Each contained sufficient enzyme tickets and ampoules of bromine water for 100 tests.



SUMMARY

The design, development, and evaluation of a pesticide detection kit for testing aqueous pesticide waste are reported. It was demonstrated that cholinesterase impregnated test tickets could be used for detection of organophosphate and carbamate pesticides in the U.S. Army Medical and Bioengineering Research and Development Laboratory filtration system. Ten pesticide detection kits were prepared and delivered to the sponsor for testing under field conditions.

Computerized and manual literature searches revealed that only cholinesterase inhibition methods are available for the rapid detection of organophosphorus and carbamate pesticides in the field. No references describing techniques for detection of chlorinated hydrocarbons that met all requirements were found.

Comparison of the bimolecular reaction rate constants with the enzyme ticket detection limits gave a statistically significant correlation ($\alpha < 0.05$) for a power relationship between the two variables.

Use of horse serum cholinesterase was precluded because of failure during storage at 40°C. The eel cholinesterase test tickets were stable for 8 months in storage at 4°C and 40°C.

No significant interference with the enzyme test was produced by solubilizing agents contained in pesticide formulations. Of 18 metal ions tested at environmentally important concentrations, only mercury(II) caused noticeable interference.

Several methods were designed to increase sensitivity of the tickets to the pesticides. Sensitivities to the phosphorothionates diazinon, malathion, and Dursban® were greatly enhanced by prior treatment with bromine water. Detection limits for all the pesticides in water were below 10 ppm.

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I. INTRODUCTION

To provide capabilities for compliance with current and future state and federal guidelines for effluent pesticide wastes, the U.S. Army Medical and Bioengineering Research and Development Laboratory (USAMBRDL) is investigating filtration techniques for treatment of wastes generated by Army installation pest control facilities. It is anticipated that these wastes will be filtered through a series of carbon columns to remove pesticide contaminants. The operator of the waste treatment facility should have some simple method for detecting harmful levels of pesticides in the filtered water prior to its release into the environment.

Midwest Research Institute (MRI) has nearly completed development of a simple technique using enzyme impregnated test tickets for the detection of nerve agents in water under the sponsorship of the U.S. Army Armament Research and Development Command (Contract No. DAAA15-76-C-0132). The test tickets are composed of two 1/2 in. diameter paper discs sealed to a thin plastic support (Figure 1). One disc is impregnated with the enzyme cholinesterase while the other contains a chromogenic substrate for the enzyme. During a test, the enzyme disc is exposed to the water to be tested, then the ticket is folded to provide contact between the enzyme and substrate discs during a short incubation. In the absence of nerve agent, a blue color is produced. When the water is contaminated by a threshold or higher concentration of nerve agent, however, the enzyme is inhibited and no color is formed.

Since organophosphate and carbamate pesticides also inhibit cholinesterase, it seemed likely that the test tickets would be applicable for the detection of these pesticides in water at pesticide filtration facilities. The objective of the current program was the modification and testing of cholinesterase test tickets for use in the USAMBRDL filtration system.

II. LITERATURE SEARCH

The goal of the literature search was two-fold. First, information pertaining to simple, rapid methods for detection of organochlorine pesticides in water which could be applied in a field-test environment was sought. Second, references supporting the background and early development of cholinesterase inhibition methods were desired by the sponsor. The literature search has been completed as originally scheduled and the findings are summarized below. All the citations retrieved by computerized methods were given in full in Monthly Technical Progress Reports Nos. 1 and 2. The most pertinent references located by other methods are cited in this report.

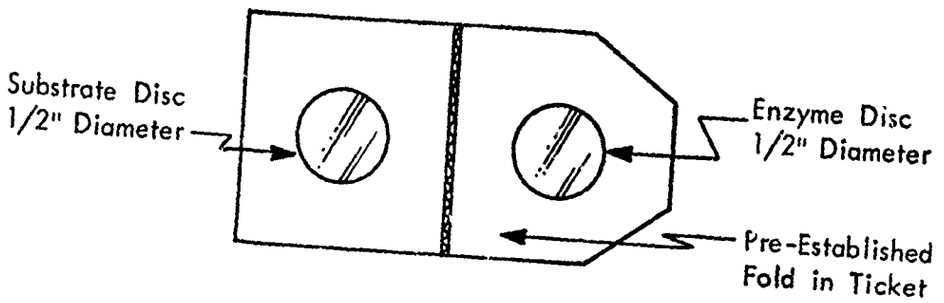


Figure 1A - Enzyme Ticket without Overlay

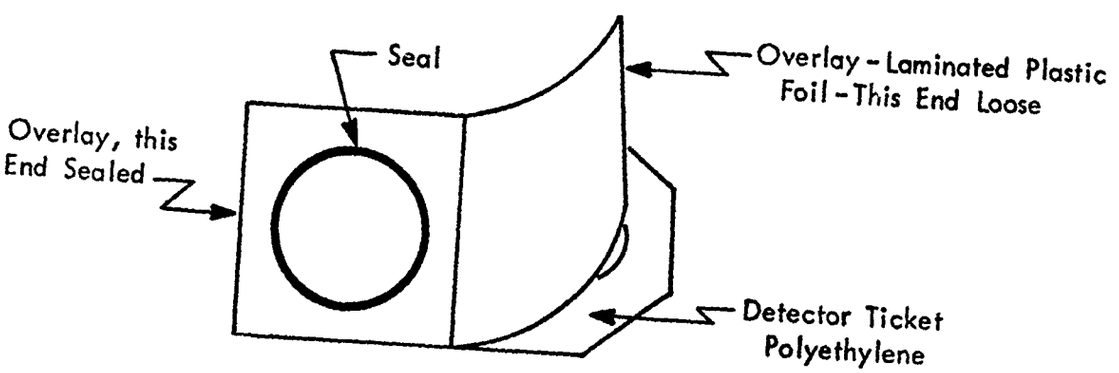


Figure 1B - Enzyme Ticket with Overlay

A. Literature Search Strategy

A minor amount of the information gathering portion of this project was done manually by searching reprints on file in our laboratories and current journals and books available in the MRI library, and by screening the subject index of the weekly Life Sciences edition of Current Contents using the key words listed in Table 1.

TABLE 1

KEY WORDS USED FOR MANUAL LITERATURE SEARCH

Carbamate

Carbaryl

Chlorinated hydrocarbon

Chlorpyrifos

Diazinon

Insecticide

Malathion

Organochlorine

Organophosphate

Organophosphorus

Pesticide

Propoxur

A much greater number of scientific journals can be scanned for pertinent articles in a short time using computerized search methods. Although many computerized literature searching services are available to MRI, it was felt that searches of Chemical Abstracts and Biological Abstracts would be most productive. Hence, search strategies for each of these data banks were designed to maximize the number of appropriate citations retrieved. The strategies consisted of three columns of terms for both Chemical Abstracts (Table 2) and Biological Abstracts (Table 3). Citations were not retrieved unless their titles or key word lists contained an item from each of the three columns.

TABLE 2

LISTS OF TERMS USED FOR COMPUTERIZED SEARCH OF CHEMICAL ABSTRACTS

Set 1	Set 2	Set 3
Insecticide(s)	Detect?[a]	Rapid
Pesticide(s)	Monitor?	Fast
Organophosphate(s)	Determin?	Quic?
Organophosphorus	Detn	Spot test(s)
Organophosphorous	Analysis	Real-time
Carbamate(s)	Analyz?	Inexpensive
Organochlorine(s)		Field test(s)
Chlorinated hydrocarbon(s)		Portable
RN[b] = 2921-88-2 (Dursban®)		
RN = 121-75-5 (malathion)		
RN = 63-25-2 (carbaryl)		
RN = 114-26-1 (Baygon®)		
RN = 333-41-5 (diazinon)		

a/ ? = Truncation symbol.
b/ RN = Chemical Abstracts Service Registry Number.

TABLE 3

LIST OF TERMS USED FOR COMPUTERIZED SEARCH OF BIOLOGICAL ABSTRACTS AND BIORESEARCH INDEX (BIOSIS)

Set 1	Set 2	Set 3
Malathion	Analysis	Speed
Carbaryl	Analyzer	Rapid
Diazinon	Assess	Fast
Dursban®	Determine	Quick
Baygon®	Monitor	Spot Test
Sevin	Monitoring	Real Time
Organophosphorus	Observation	Inexpensive
Organochlorine	Detect	Field Test
Carbamate	Detn	Portable
Chlorinated Hydrocarbon		

B. Results of Literature Search

The computerized literature searches retrieved a total of 202 citations (see Table 4). Nearly all the referenced articles were secured from local libraries and carefully examined by the principal investigator. Fifty-five of the articles were written in a foreign language, usually Japanese or Russian, and were not translated although several articles provided abstracts or summaries in English. Fifty-nine of the references dealt with gas chromatographic (GC) methods for analysis of pesticides, or with techniques for sampling, sample extraction, or sample cleanup prior to GC analysis. References dealing with analyses based on chemical methods, cholinesterase inhibition, thin-layer chromatography (TLC), and high performance liquid chromatography (HPLC) accounted for about 10% of the total. The BIOSIS search produced a surprisingly high number (61) of irrelevant articles. These deal with such varied topics as pesticide metabolism, efficacy of pesticides on insect populations, and the use of carbamates for analysis of nickel.

TABLE 4

DISTRIBUTION OF COMPUTER-RETRIEVED CITATIONS BY TOPIC

	CHEM 77-78	CHEM 72-76	BIOSIS	TOTAL
Foreign Language[a/]	14	31	10	55
Gas Chromatography[b/]	10	19	30	59
Chemical	1	4	1	6
Cholinesterase	1	4	3	8
TLC[c/]	0	4	0	4
HPLC[d/]	0	0	5	5
Irrelevant	0	4	61	65
Total	26	66	110	202

a/ Generally Japanese or Russian.
b/ Including methods for sampling, extraction, and clean-up.
c/ Thin-layer chromatography.
d/ High performance liquid chromatography.

1. References dealing with organochlorine pesticide analysis: Nearly all the articles which addressed analysis of organochlorine pesticides used gas chromatography as the analytical tool. In fact, gas chromatography is recommended as the method of choice in Standard Methods for the Examination of Water and Wastewater, and in state-of-the-art reviews from the International Union of Pure and Applied Chemists and the Department of Health, Education, and Welfare.[1-3] Methods which employ gas chromatographic analyses would not normally be applicable in pesticide filtration facilities due to the low portability of the equipment. Thus, the standard, nearly universally accepted procedure for analysis of chlorinated pesticides is not applicable for this use. However, a portable gas chromatograph, manufactured by Analytical Instrument Development, Inc., has been reported and is claimed sensitive to 0.1 ppm of heptachlor, malathion, aldrin, heptachlor epoxide, dieldrin, endrin, and DDT.[4] Although this device can probably meet sensitivity and selectivity requirements, its cost, which was not given in the article, may be prohibitive for use in a large number of pesticide filtration plants.

All the standard chemical methods described in the retrieved articles were based on some property of the pesticide which made it react in a characteristic way to give a readable indication, usually a color change. Each of these methods had some apparent disadvantage for the present use, such as insufficient sensitivity or selectivity.

None of the cholinesterase methods were applicable for detection of chlorinated hydrocarbons. HPLC, like GC, suffers from high expense and low portability.

Three of the four TLC methods cited were specific for anticholinesterase pesticides. One TLC technique, however, was designed for the analysis of organochlorine insecticides in blood.[5] This method has the advantages of being quite sensitive (a detection limit of 0.01 ppm in blood, under the right conditions, was claimed) and the chromatography step, which might be improved by performing chromatography in the second dimension, provides some selectivity. However, the method is time-consuming ($\sim$ 30 min for an eight-sample analysis), demands a considerable array of chemicals and apparatus, and requires the availability of ultraviolet light (110 v AC) for visualization of spots.

It is conceivable that some sort of enzyme inhibition method could be employed to better visualize the chromatographic spots and simplify the procedure. Mendosa et al. have developed a similar system for anticholinesterase pesticides using a TLC plate impregnated with a chromogenic substrate and a developing spray containing active cholinesterase.[6-8] Pesticides are visualized as white spots on a colored background. In an analogous procedure, organochlorine pesticides might be separated by TLC. The TLC plate would then be sprayed with an enzyme which is specifically inhibited by chlorinated

pesticides. After a short incubation, active enzyme could be made visible by spraying the plate with a chromogenic substrate. The background would become colored due to the action of the enzyme on the substrate while no color production would occur within the pesticide spots.

Apart from this speculative TLC method, no simple method for the rapid detection of chlorinated hydrocarbon pesticides outside a laboratory environment has been found.

2. References concerned with development of cholinesterase inhibition methods: Contemporary laboratory analyses of organophosphate and carbamate pesticides are generally accomplished using standard methods of GC and HPLC.[9-10] However, aside from the portable GC mentioned previously and a few isolated, direct chemical methods which suffer from poor sensitivity or selectivity, only the cholinesterase inhibition methods appear suitable for detection of organophosphate and carbamate pesticides in the field.

Excellent reviews of the molecular properties, mechanisms of catalysis and inhibition, and biological functions of acetylcholinesterase are available.[11-13] Reviews have also been written on cholinesterase inhibition methodologies for detection and quantitation of anticholinesterase agents using a variety of approaches.[10-14] However, much of the pioneering work in the development and application of the cholinesterase inhibition technique for real-time and spot-test analysis of pesticides in the workplace and the environment was performed at MRI by Dr. Louis H. Goodson and Mr. William B. Jacobs. Several of their more pertinent publications are listed in the bibliography.[15-27] Their efforts have resulted in cholinesterase-based analytical techniques that range in sophistication from real-time monitors that automatically analyze water for pesticides at the parts per million level to the simple, cholinesterase-impregnated tickets that are the subject of the current investigation. The actual development of the cholinesterase test tickets has been supported by the U.S. Army and reported in technical progress reports rather than published in the open literature.[28-31] A careful perusal of this list of publications and reports will show that cholinesterase techniques can, in certain cases, be as sensitive as GC and HPLC, in other cases more portable, and nearly always simpler and less expensive than the classical methods of analysis.

III. QUANTITATIVE ANALYSIS OF PESTICIDE SOLUTIONS

During the early portions of this program it was decided to prepare working stock solutions of the five pesticides of interest at concentrations around 500 ppm (w/v) in methanol and to dilute these with water as necessary to provide solutions for ticket testing. It was felt that methanol was a better solvent than water for stock solutions because of better

solubilities of most of the pesticides in methanol, the elimination of the need for extracting pesticide-water solutions prior to GC analysis, and a suspected increase in stability of some of the pesticides in methanol. It was therefore necessary to periodically analyze the methanolic stock solutions to demonstrate the stability of the pesticides in methanol.

Each of the solutions were analyzed according to conventional quantitative procedures recommended by USAMBRDL. Diazinon, Dursban®, and malathion stock solutions were analyzed by gas chromatography (GC) while Baygon® and carbaryl were estimated by high performance liquid chromatography. (HPLC). These pesticides were identified by the sponsor as the anticholinesterase insecticides most often used on Army installations and, as such, they became the test pesticides for the entire program.

A. Gas Chromatographic Analysis of Diazinon, Dursban®, and Malathion

1. Pesticide standards for gas chromatography: Samples of malathion, Dursban®, and diazinon as well as Baygon® and carbaryl were obtained from the Quality Assurance Section, Analytical Chemistry Branch, EPA, HERL, ETD (MD-69), Research Triangle Park, North Carolina 27711, and were used as analytical reference standards. One-gram samples of the same pesticides were purchased from Chem Service, Inc., 304 Turner Lane, P.O. Box 194, West Chester, Pennsylvania 19380. These samples were used routinely during the project to make stock pesticide solutions. The exact concentrations of the stock solutions were determined by gas chromatography or HPLC and comparison with standard curves prepared with the analytical reference standards from EPA.

2. Procedure for pesticide standard curves: Liquid pesticide standards were diluted by adding 8 to 11 µl of the EPA standard pesticide to a tared 10 ml volumetric flask containing 9 ml of methylene chloride, reweighing, and diluting to the mark. Solids were weighed in a tared test tube, dissolved in methylene chloride, and transferred to a 10 ml volumetric flask followed by dilution to the mark. Portions of these standards were diluted to give four solutions of each pesticide with concentrations between about 100 ppm (w/v) and 1,000 ppm (w/v).

Two microliters of each solution were injected into the gas chromatograph at an injection port temperature of 250°C. The samples were chromatographed on a 6 ft by 4 mm ID column of 3% OV-17 on Gas Chrom Q at a column temperature of 200°C. Effluent pesticides were detected using a flame ionization detector (FID) at 250°C. The flame was maintained with pressurized air and hydrogen. Carrier gas was nitrogen at 60 ml/min.

Peak heights were normalized according to instrument attentuation then plotted against pesticide concentration (Figure 2). The Dursban® and

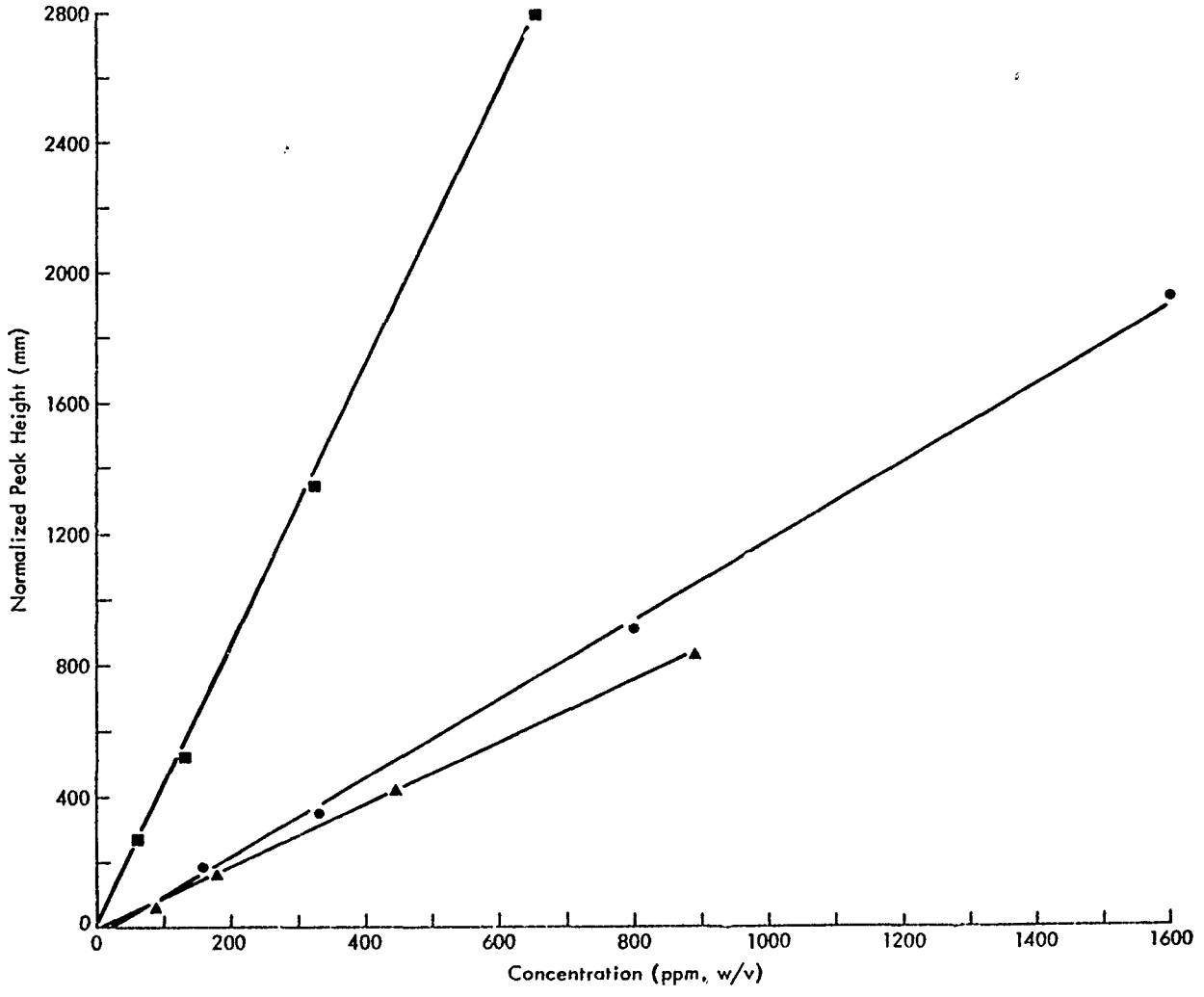


Figure 2 - Gas Chromatographic Standard Curves for Diazinon, Dursban®, and Malathion. Chromatography was performed with a 6 ft x 4 mm ID column of 3% OV-17 on Gas Chrom Q at 200°C using nitrogen at 60 ml/min as carrier gas and a flame ionization detector. Diazinon, squares: Dursban®, circles; malathion, triangles.

malathion reference standards received from EPA were claimed by this source to be more than 99% pure, so no correction of concentration was used for these compounds. The purity of the diazinon standard, however, was stated as only 87.4%, so each apparent diazinon concentration was multiplied by 0.874.

3. Results of gas chromatographic standard curves for diazinon, Dursban®, and malathion: The stock solutions of diazinon, Dursban®, and malathion were analyzed at approximately monthly intervals. Results in Table 5 show that no trend was apparent in concentrations of diazinon or

TABLE 5

GAS CHROMATOGRAPHIC ANALYSIS OF METHANOL STOCK SOLUTIONS OF DIAZINON, DURSBAN® AND MALATHION

Pesticide	Date of Analysis	Standard Curve Data			Stock Solution Concentration From GC Analysis (ppm [w/v])[b/]	Stock Solution Concentration From Original Weighing (ppm [w/v])[c/]
		r^2[a/]	Intercept (mm)	Slope (mm/ppm)		
Diazinon	11/13/78	0.9993	27	3.0	470	521
	11/20/78	0.9991	41	2.9	513	
	12/6/78[d/]	0.9994	-4	3.6	479	
Dursban®	11/20/78[e/]	0.9989	5	0.9	777	625
	12/6/78	0.9983	5	1.1	664	
	1/12/79	0.9999	-3	1.1	550	
Malathion	11/13/78	0.9996	-20	0.7	597	629
	11/20/78	0.9984	3	0.6	621	
	12/6/78	0.9982	11	0.8	628	
	1/12/79	0.9975	-11	0.9	520	

a/ Correlation coefficient squared.

b/ Estimated concentration of pesticide stock solution using GC analysis and appropriate standard curve.

c/ Calculated concentration of pesticide stock solution obtained from the weight of pesticide and the volume of solution.

d/ No data available for 1/12/79 since diazinon stock solution was previously expended.

e/ Dursban stock solution analysis on 11/13/78 deleted due to probable dilution errors during preparation of solutions for standard curve.

malathion for at least 30 and 60 days, respectively, suggesting good stability of these pesticides in methanol. The variability noted in the stock solution concentrations for diazinon and malathion is unexplained. Although we do not have a backlog of experience to give us an idea of expected variability in diazinon and malathion stock solution concentrations as determined by GC, other analytical experience implies that this variability is excessive and probably due to an operator procedural error such as improper dilution or calculation, solvent evaporation, or incorrect pipetting technique. It seems apparent, however, that these variations are not masking a rapid and significant decomposition of these pesticides in methanol. A marked decrease in Dursban® concentration was noted over a 53-day period (11/20/78 to 1/12/79). These results indicate that Dursban may be relatively unstable in methanol.

B. High Performance Liquid Chromatographic (HPLC) Analysis of Carbaryl and Baygon® Methanolic Stock Solutions

1. Procedure for HPLC analyses: The stock solutions of carbaryl and Baygon® were analyzed according to the HPLC procedure received from USAMBRDL. A Waters' component liquid chromatography system was used which consisted of two M6000A pumps, a 660 programmer, a U6K injector, a 440 UV detector, and a Heath recorder. The operating parameters were:

	Carbaryl	Baygon®
Column:	Waters' μPak C_{18}, 30 cm x 4 mm	Waters' μPak C_{18}, 30 cm x 4 mm
Solvent:	60% MeOH/H_2O	50% MeOH/H_2O
Flow rate:	1 ml/min	1 ml/min
Chart speed:	10 min/in.	10 min/in.
Detection:	254 nm, 0-1.0 A	254 nm, 0-0.1 A
Injection volume:	10 μl, 15 μl	15 μl

Duplicate injections of standards and stock solutions were used throughout.

To provide additional information about these pesticide solutions, an injection of both the carbaryl EPA reference standard and the carbaryl stock solution were made at an expanded scale. No extra peaks greater than 0.1% of the main peak were seen in either chromatogram. A similar treatment of the Baygon® solutions, however, revealed three additional minor peaks. The data in Table 6 imply that the stock Baygon® solution has lower amounts of these impurities than does the EPA reference.

TABLE 6

HPLC SCAN FOR TRACE COMPONENTS IN BAYGON® REFERENCE STANDARD AND STOCK SOLUTIONS

Sample	Peak Height (mm)			
Baygon® Reference	15 (0.5%)	2,960 (97.7%)	17 (0.6%)	36 (1.2%)
Baygon® Stock	19 (0.6%)	2,940 (98.7%)	13 (0.4%)	9 (0.3%)
Retention Volume	3.5	5.5	7.0	10.5

2. Results of HPLC analyses of pesticide stock solutions: Results given in Table 7 show that apparent concentrations of both carbaryl and Baygon® increased markedly over the 58-day period between 11/27/78 and 1/24/79. The reason for this increase is not known although solvent evaporation has not been ruled out. The lack of significantly decreasing concentrations between analyses as well as the absence of any new peaks on comparative HPLC scans at 10-fold scale expansion during either analysis is good evidence that these pesticides are not decomposing at a pronounced rate.

TABLE 7

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF METHANOLIC STOCK SOLUTIONS OF CARBARYL AND BAYGON®

Pesticide	Calculated[a/] 10/30/78 (ppm, w/v)	HPLC Analysis 11/27/78 (ppm, w/v)	HPLC Analysis 1/24/79 (ppm, w/v)
Carbaryl	688	736	789
Baygon®	486	492	553

a/ Calculated from weight of pesticide and solution volume at time of solution preparation.

IV. REACTION RATE CONSTANTS FOR CHOLINESTERASE INHIBITION BY PESTICIDES

Cholinesterase is inhibited by organophosphate and carbamate pesticides in a bimolecular reaction that is essentially irreversible. Incubation of enzyme samples with increasing concentrations of pesticide produces exponentially decreasing enzyme activities under appropriate conditions. This relationship, when used as a standard curve, allows determination of the concentration of a solution containing the organophosphate or carbamate pesticide for which the curve was prepared if no other cholinesterase inhibitors are present. Results from the standard curves can also be used to calculate rate constants for the bimolecular reaction between enzyme and pesticide in solution. These rate constants, it was theorized, might be related to the rate of reaction between the pesticide and the enzyme immobilized in the test tickets. If this relationship were consistent for the five pesticides of interest in this program, then it might be expected to hold true for other pesticides. If this proved to be the case, it would be possible to determine a solution rate constant for reaction between the enzyme and some new pesticide, and from this rate constant predict the sensitivity of the enzyme ticket to the new pesticide. This would eliminate the necessity of extensive sensitivity testing of the tickets with each new pesticide.

The goal of this portion of the program was to determine the reaction rate constants for cholinesterase inhibition by the pesticides. The derivation of an empirical relationship between these rate constants and the enzyme ticket detection limits for each pesticide is presented in Section V-B. The following sections contain a description of the procedures for analysis of enzyme activities, an explanation of the method for calculation of rate constants from standard curve data, and a discussion of the results.

A. Procedures for Enzyme Analyses

Two forms of cholinesterase, one extracted from electric eel and the other obtained from horse serum, are in routine use in our laboratories. Since the enzymes show varying sensitivities to previously studied inhibitors, the rate constants for the reaction of each enzyme with each of the five pesticides of interest were determined during this program.

The method for the determination of horse serum cholinesterase activity is modified from a procedure obtained from Sigma Chemical Company, St. Louis, Missouri, while that for electric eel cholinesterase was taken from Ellman et al.[33] The two methods are identical except that the method for the horse serum enzyme uses butyrylthiocholine iodide as substrate whereas the eel enzyme uses acetylthiocholine iodide as substrate.

Test tubes are preincubated at 37°C in a shaking water bath. One milliliter of the enzyme solution (about 0.33 unit horse serum or 0.5 unit eel cholinesterase per milliliter of pH 7.4, 0.08 M THAM (Tris[hydroxymethyl]-aminomethane) buffer is incubated in a tube in the bath for 2 min. One milliliter of the water sample containing pesticide is mixed with the enzyme and 1 ml of the mixture is pipetted into a cuvette previously thermally equilibrated in a spectrophotometer and containing 0.02 ml of the appropriate substrate, 0.1 ml of 5,5'-dithiobis(2-nitrobenzoic acid) (a chromogenic thiol reagent), and 2 ml of THAM buffer. The linear rate of change of absorbance at 410 nm is recorded, calculated, and multiplied by a conversion factor to give the enzyme specific activity (units/milligram) as follows.

$$\text{Enzyme specific activity (units/mg)} = (\Delta A/\text{min}) \times (\text{enzyme factor})$$

where $\Delta A/\text{min}$ is the linear rate of change of absorbance, and enzyme factor is a constant for each enzyme solution and is expressed in units of activity units·min/mg·ΔA from this equation:

$$\text{Enzyme factor} = \frac{(0.4588) \times (\text{ml enzyme stock})}{\text{mg enzyme}}$$

With practice, analyses of water samples can be performed at a rate of about 20 analyses/hr/operator.

B. Calculation of Rate Constant from Standard Curve Data

In vitro incubation of cholinesterase with organophosphate or carbamate pesticide for a specific time interval (e.g., 4 min) results in a decreased cholinesterase activity as measured by the spectrophotometric assay method. By varying the concentration of pesticide and analyzing the resultant enzymatic activity, a nonlinear standard curve can be constructed with activity as the ordinate and pesticide concentration as the abscissa (Figure 3). Unknown concentrations of pesticide can be estimated by measuring cholinesterase activity after incubation with the unknown solution, then projecting this activity onto the standard curve and interpolating to find pesticide concentration. However, the manual calculation portion of the procedure, that is, calculation of enzyme activities, plotting of the standard curve, and interpolation of unknown pesticide concentrations, is time-consuming and subject to error.

A substantial improvement in the calculation has been developed in our laboratories and is due to the observation by us and by others that the

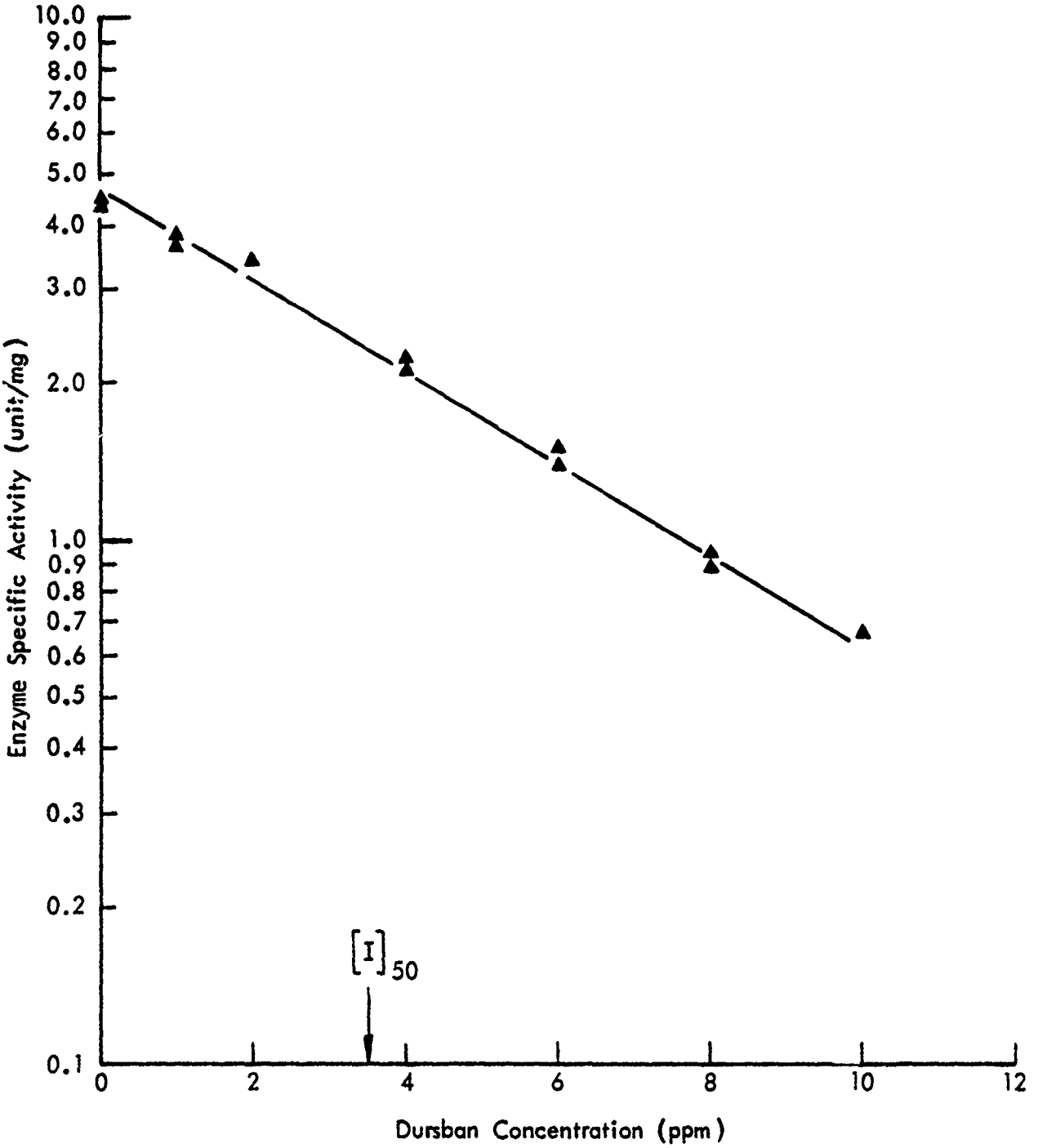


Figure 3 - Example of Standard Curve for the Inhibition of Horse Serum Cholinesterase by Dursban® after a 4-min Incubation at 37°C. $[I_{50}]$ denotes the concentration of inhibitor which results in inhibition of 50% of the enzyme under these conditions. $r^2 = 0.99565$, $a = 4.74$ units/mg, and $b = -6.96 \times 10^4\ M^{-1}$.

enzymatic activity decreases exponentially with increasing inhibitor concentration. Thus, the following equation holds:

$$Y = ae^{bx} \tag{1}$$

where Y is the observed enzymatic activity, x is the molar concentration of pesticide standard, and a and b are constants. This exponential relationship is consistent with a bimolecular reaction mechanism observed under conditions which yield pseudo-first order kinetics. Regression and correlation of the linear form of this equation

$$\ln(Y) = \ln(a) + bx \tag{2}$$

using data from over 500 previously run eel cholinesterase standard curves have yielded squared correlation coefficients (r^2) which are always greater than 0.96.

It was demonstrated in Monthly Technical Progress Report No. 1 that the constant a is equal to the enzyme activity in the absence of inhibitor and that the bimolecular reaction rate constant k is equal to the negative of the constant b divided by the time in minutes of enzyme-inhibitor incubation.

$$k = -b/t$$

Thus, reaction rate constants can be determined from cholinesterase inhibition standard curves.

C. Inhibition Rate Constants

The data in Table 8 indicate the relative sensitivities of cholinesterase from horse serum and electric eel to inhibition in solution by the five pesticides of interest. Interestingly, the horse serum enzyme shows roughly equivalent sensitivities to Dursban®, Baygon®, carbaryl and diazinon as evidenced by bimolecular reaction rate constants that are about the same order of magnitude, while the eel enzyme shows sensitivities that vary by about 10^3. Both enzymes were very insensitive to malathion. In fact, preliminary experiments showed no inhibition of horse serum cholinesterase by 2,000 ppm malathion. This lack of sensitivity for malathion relative to the other pesticides is not surprising in view of the relatively low toxicity of this compound.

TABLE 8

BIMOLECULAR REACTION RATE CONSTANTS FOR INACTIVATION OF EEL AND HORSE CHOLINESTERASE BY SEVERAL PESTICIDES

Pesticide	Enzyme Source[a]	r^2[b]	b[c] (M^{-1})	k[d] ($M^{-1}min^{-1}$)	$[I_{50}]$[e] (M)	$[I_{50}]$ (ppm)
Dursban®	Horse	0.99565	-6.96×10^4	1.74×10^4	9.96×10^{-6}	3.49
		0.99360	-8.33×10^4	2.08×10^4	8.32×10^{-6}	2.92
	Eel	0.96006	-2.18×10^3	5.44×10^2	3.18×10^{-4}	1.11×10^2
		0.99147	-2.55×10^3	6.38×10^2	2.71×10^{-4}	9.50×10^1
Baygon®	Horse	0.97808	-7.15×10^4	1.79×10^4	9.69×10^{-6}	2.03
		0.96119	-6.29×10^4	1.57×10^4	1.10×10^{-5}	2.30
	Eel	0.99526	-3.76×10^6	9.40×10^5	1.84×10^{-7}	3.85×10^{-2}
		0.99676	-3.98×10^6	9.95×10^5	1.74×10^{-7}	3.64×10^{-2}
Carbaryl	Horse	0.99408	-2.07×10^4	5.17×10^3	3.35×10^{-5}	6.75
		0.99155	-1.59×10^4	3.97×10^3	4.36×10^{-5}	8.77
	Eel	0.99545	-1.81×10^5	4.53×10^4	3.82×10^{-6}	7.69×10^{-1}
		0.98495	-1.74×10^5	4.36×10^4	3.98×10^{-6}	8.00×10^{-1}
Diazinon	Horse	0.99543	-7.83×10^4	1.96×10^4	8.85×10^{-6}	2.69
		0.99123	-4.03×10^4	1.01×10^4	1.72×10^{-5}	5.23
	Eel	0.98831	-3.52×10^3	8.79×10^2	1.97×10^{-4}	6.00×10^1
		0.98769	-2.79×10^3	6.98×10^2	2.48×10^{-4}	7.56×10^1
Malathion[f]	Horse	-	-	-	-	-
		-	-	-	-	-
	Eel	0.96904	-1.64×10^3	4.09×10^2	4.24×10^{-4}	1.40×10^2
		-	-	-	-	-

a/ Enzyme was either horse serum cholinesterase or electric eel acetylcholinesterase.

b/ r^2 is the correlation coefficient squared.

c/ b is a constant from the standard curve.

d/ k is the bimolecular rate constant for the inactivation of cholinesterase by pesticide.

e/ $[I_{50}]$ is that concentration of inhibitor which results in 50% inhibition. Results in this table were obtained after 4 min incubation at 37°C.

f/ Horse serum cholinesterase was not inhibited by up to 2,000 ppm malathion. Only one standard curve was performed with malathion and eel enzyme since pesticide solutions were cloudy above about 300 ppm and the curve showed deviation from exponential.

V. ENZYME TICKET DETECTION LIMITS TESTING

In order for the enzyme impregnated test tickets to be applicable for testing pesticide filtration plant water, it is important that the minimum pesticide concentrations at which the tickets will be completely inhibited be well defined. The minimum concentration for detection of a particular pesticide is termed its "detection limit." At concentrations below but near the detection limit, however, the test loses its all-or-none character and the amount of color formed is dependent upon the concentration of inhibitor. Generally, plotting the average color intensity produced with the enzyme test tickets against concentration of inhibitor yields a reversed J-shaped curve with a gradual transition from completely active to completely inhibited enzyme. At very low concentrations of inhibitor, no inhibition is noted and all tests give maximal color intensities. As inhibitor concentration is increased, the average color intensity produced decreases until at some inhibitor concentration no color is formed at all. This concentration is defined as the detection limit for the test tickets.

A. Determination of Pesticide Detection Limits

1. Enzyme ticket preparation procedure: The enzyme tickets which are the topic of this program are being developed under a separate contract (Contract No. DAAA15-76-C-0132) with the U.S. Army for the detection of nerve agents in water. The detailed procedure for the preparation of eel and horse serum cholinesterase and substrate discs is given in the Appendix to this report. All cholinesterase tickets tested in this program were prepared according to this procedure.

2. Enzyme ticket test procedure: All ticket tests were performed according to a set procedure. A ticket was removed from its sealed packet and the enzyme disc was dipped in the sample for 15 sec. This time allows complete wetting of the enzyme disc and absorption of the inhibitor. The ticket was incubated for 15 min at room temperature to provide sufficient time for the enzyme-inhibitor reaction to take place. The substrate overlay was then removed to expose the substrate disc and the two paper discs were held in contact between thumb and forefinger for 2 min. The color and intensity of the enzyme disc was then determined by comparison with the standard Munsell color chips.

These color standards were obtained from the Kollmorgen Corporation, Baltimore, Maryland. Color intensity ratings of 0, +1, +1.5, +2, +3 and +4 were assigned to columns of graded color standards to allow mathematical data reduction. It should be emphasized that the color standards and the color intensity scale are not to be used in the field. In actual use, the operator will be instructed only to examine the enzyme disc for the presence (negative test) or absence (positive test) of color.

Generally, five or ten tickets have been tested at each pesticide concentration and experimental condition. Color intensities were averaged and standard deviations were calculated. Graphs in this report show these average values with "tails" that indicate $\pm$ one standard deviation.

3. Elimination of horse serum cholinesterase: The experimental plan of this portion of the program included testing as many of the five pesticides with both horse and eel tickets as possible and, as data accumulated, to eliminate either the eel or horse tickets as soon as possible. This was desirable since only one ticket type would be submitted for use at pesticide filtration plants and early elimination from testing of the other ticket type would save unnecessary effort.

Tickets made with horse serum cholinesterase were removed from further testing for three reasons. First, as will be shown in Section VI, horse serum tickets are relatively unstable during storage at 40°C. Second, the horse serum enzyme was found to be less sensitive, as measured by inactivation rate constants, than eel cholinesterase to inhibition in solution by three of the five pesticides of interest (Section IV-C). Finally, three of the pesticides are not sufficiently soluble alone in water to determine their detection limits with enzyme tickets, and 1% Triton X-100 had been selected as the solvent for detection limit determinations. As shown in Section V-C-1a, horse serum cholinesterase tickets are inhibited by as little as 0.04% Triton X-100.

4. Detection limits: Detection limits were determined using eel cholinesterase tickets (1.2 units/disc) prepared following the procedure in the Appendix. Each of the five pesticides was dispersed in 1% Triton X-100. In addition, commercially available formulations for four of the pesticides were acquired and tested. All pesticide preparations in water or 1% Triton X-100 were clear and free of obvious precipitate. All pesticide formulations diluted with water were milky with no visible settling with time.

The inhibition curve shown in Figure 4 for Baygon® in water is typical of the other pesticides and solvents. At low concentrations of pesticide, no inhibition is evident and +3 color intensities were uniformly produced. At higher pesticide concentrations, increased inhibition was evidenced by lower color intensities. Pesticide detection limits were taken as the lowest concentration of pesticides that would consistently cause complete enzyme inactivation -- that is, no color formed in five out of five trials.

The results for all the pesticides are summarized in Table 9. Detection limits of 2 and 9 ppm (w/v) for Baygon® and carbaryl are low enough to be of value for monitoring in the field. However, the sensitivities of the tickets to inhibition by the other three pesticides even in the formulated products were much poorer. Efforts to improve their detection

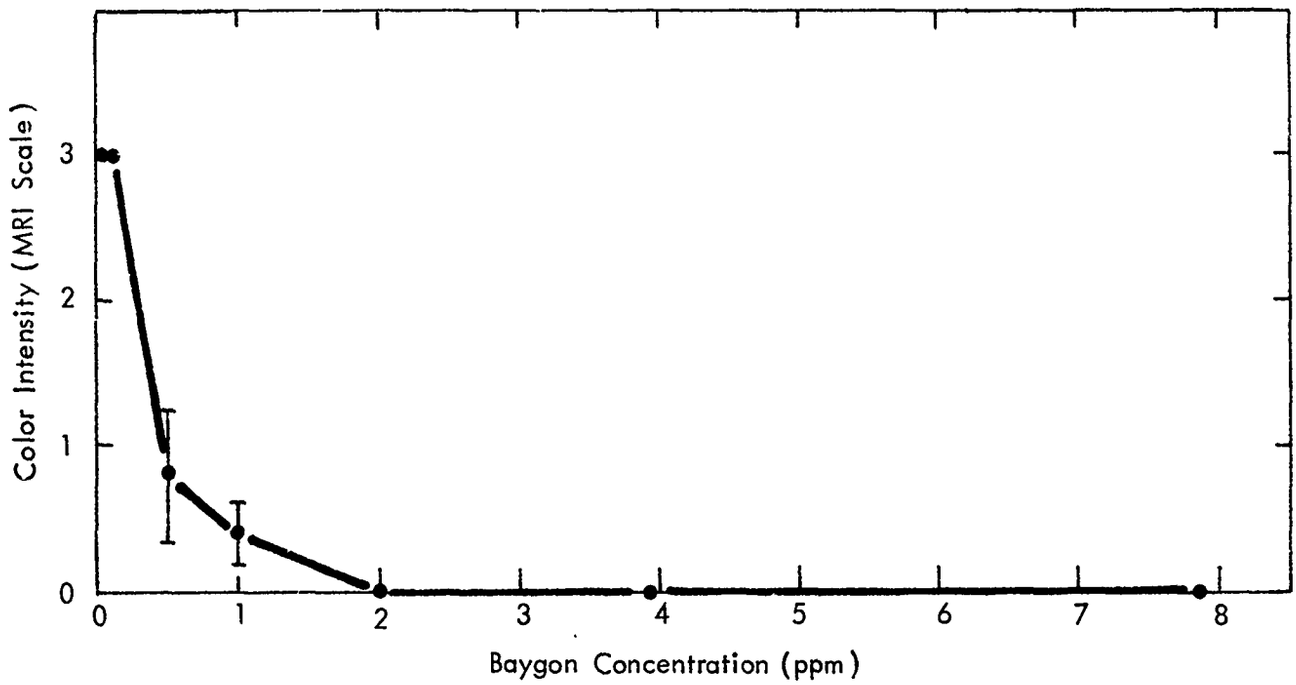


Figure 4 - Color Production with Eel Cholinesterase Tickets (1.2 units/disc) after Inhibition with Several Concentrations of Baygon® in Water

Points represent average color intensity observed with five trials. Vertical lines show ± 1 standard deviation.

limits using bromine oxidation have been successful. These results are discussed in Section V-D.

TABLE 9

TICKET DETECTION LIMITS FOR FIVE PESTICIDES

	Detection Limit (ppm, w/v)[a]			
	Pure Pesticide		Formulated Pesticide	
Pesticide	in Water	in 1% Triton X-100	in Water	in 1% Triton X-100
Baygon®	2	2	- [c]	- [c]
Carbaryl	9	9	9	-
Diazinon	- [b]	100	400	-
Dursban®	- [b]	2,000	300	600
Malathion	- [b]	2,500	100	-

a/ Detection limit is the lowest concentration of pesticide that gives complete inhibition of enzyme impregnated tickets. Eel cholinesterase tickets (1.2 units/disc) were prepared according to procedures given in the Appendix.

b/ Not sufficiently soluble to determine detection limit.

c/ Formulated Baygon® not tested during this experiment. Detection limit in water later determined to be 2 ppm (w/v).

B. Relationship Between Inhibition Rate Constants in Solution and Ticket Detection Limits

It has been pointed out that rate constants for inactivation of cholinesterase in solution were determined to develop a mathematical model that would describe the relationship between the rate constants and the enzyme ticket detection limits. Such a relationship might be used to predict enzyme ticket detection limits of new pesticides based on their inhibition rate constants.

Examination of the data in Table 10 shows that arranging the five pesticides in order of decreasing rate constant also places them in order of increasing detection limits in 1% Triton X-100. Thus, there appears to be a relationship between these two quantities. Correlations between rate constants and detection limits were not statistically significant for linear, exponential, or logarithmic relationships. However, the correlation was significant ($\alpha < 0.05$) for a power relationship of the form

$$Y = ax^b$$

where Y is inhibition reaction rate constant and x is detection limit. In fact, the data fit a special form of the power curve since b is close to -1 (b = -0.9923). Thus, a linear plot of this symmetrically hyperbolic function with rate constants on the Y-axis and detection limits on the X-axis would asymptotically approach the Y-axis at low values of x and asymptotically approach the X-axis at low values of Y. The physical significance, if any exists, of this relationship is unknown. Rate constants and ticket detection limits would have to be determined for another series of pesticides to evaluate the prediction accuracy of this relationship.

TABLE 10

SOLUTION INHIBITION REACTION RATE CONSTANTS AND TICKET DETECTION LIMITS FOR FIVE PESTICIDES

Pesticide	k [a] (M^{-1} min^{-1})	Detection Limit [b] (ppm, w/v)
Baygon®	9.7×10^5	2
Carbaryl	4.4×10^4	9
Diazinon	7.9×10^2	100
Dursban®	5.9×10^2	2,000
Malathion	4.1×10^2	2,500

a/ k is the bimolecular reaction rate constant for inactivation of eel cholinesterase in water at 37°C.

b/ Detection limit is the lowest concentration of pesticide that gives complete inhibition of enzyme impregnated tickets. Eel cholinesterase tickets (1.2 units/disc) were prepared according to procedures given in the Appendix. Samples of the pure pesticides were solubilized in 1% Triton X-100.

In contrast, this relationship is not evident when detection limits are determined for formulated pesticides dispersed in water. However, gross relationships still hold. Pesticides with very high rate constants (Baygon®, carbaryl) have low detection limits while pesticides with low rate constants (diazinon, Dursban®, malathion) have higher detection limits.

The mechanism by which the nonpesticide ingredients of formulations affect detection limits is unknown. The situation is further confused by noting that detection limits are higher for formulated diazinon, lower for formulated Dursban® and malathion and the same for formulated carbaryl as compared to their nonformulated counterparts.

C. Interference Testing

Compounds other than pesticides could interfere with the enzyme test in three ways: by inactivating the enzyme in some way and causing a false positive test in the absence of pesticide; by somehow protecting the enzyme from inactivation by a pesticide, thus producing a false negative test; or by acting synergistically with a small concentration of pesticide which is below its normal detection limit to give a positive test.

An experimental plan was adopted to identify these types of interferences. Tickets were tested for their abilities to form color after exposure to various concentrations of the potential interfering compound. Plotting average color intensity as a function of interfering compound concentration would show a minimum concentration necessary to cause complete inhibition, if one exists, in a manner analogous to determination of detection limits for pesticides. This procedure tested for the possibility of false positives in the absence of pesticides. Testing over a range of interfering compound concentrations was also attractive since little information was available concerning expected concentrations of these materials in the filtration plant. Performance of an inhibition curve as a function of pesticide concentration at constant interfering compound concentration showed whether the pesticide detection limit determined in the absence of interfering compound was shifted to a higher or lower concentration by the presence of the interfering compound. Displacement of the detection limit to a higher pesticide concentration indicates protection of the enzyme by the interfering compound and opens the possibility of false negative tests. A shift of the detection limit to a lower concentration of pesticide implies a synergism between pesticide and interfering compound and increases the probability of positive tests at pesticide concentrations below the normal detection limit.

1. Pesticide solubilizers: Materials which seemed to have the highest potential for interfering with the enzyme test were the solubilizers added to pesticide formulations to enhance solution or suspension of the

hydrophobic pesticides in water. These compounds were not expected to be active-site directed inhibitors of cholinesterase as are the organophosphate and carbamate insecticides. Any compound, however, which has the capability to disrupt the tertiary structure or conformation of an enzyme molecule, as many of these solubilizers do, is likely to inactivate the enzyme at sufficiently high concentrations.

a. Triton X-100: It was found early in the program that several of the pesticides of interest were very poorly soluble in water. Triton X-100 was chosen as a solubilizer for these pesticides since it is commonly used in some biochemical procedures and is a component of the eel cholinesterase impregnated discs. Before this material could be used as a pesticide solubilizer, however, its potential interference with enzyme ticket tests had to be determined. Triton X-100 concentrations as high as 1% did not interfere with color production when tested with eel cholinesterase tickets. In fact, tests indicate that good color intensity is produced by these tickets even after exposure to 10% Triton X-100. However, tickets made with horse serum cholinesterase proved to be rather sensitive to this solubilizer. It was found that concentrations of Triton X-100 as low as 0.04% (400 ppm, v/v) completely and reproducibly inhibit the enzyme. This finding effectively eliminated the use of horse serum tickets for testing pesticide suspensions in Triton X-100.

b. Methanol: It was demonstrated that methanol in water was without effect on eel tickets at concentrations up to about 10%. Higher methanol concentrations progressively caused more enzyme inhibition until complete inhibition was reached at about 40% methanol.

c. Triton X-180 and X-190: Limited testing with Triton X-180 and Triton X-190 showed that these solubilizers would probably yield complete eel ticket inactivation at 2 to 3% in water.

d. Heavy aromatic naphtha (HAN): Suspensions of 1%, 2%, and 5% HAN in 0.5% Triton X-190 did not inhibit eel cholinesterase tickets but concentrations of 10% prevented complete color formation. It was concluded that HAN suspended in water at concentrations below about 10% would not directly interfere with the enzyme ticket tests in the absence of pesticide.

Additional tests were done to determine the effect of HAN on the detection limit of carbaryl. Carbaryl was dissolved in a water suspension of 0.1% Triton X-190 and 0.1% HAN. Dilutions were made using this solvent system to prepare carbaryl test solutions. The detection limit for carbaryl under these conditions was found to be about 15 ppm. It appears, since this is close to the detection limit (9 ppm) determined in 1% Triton X-100 (Table 9), that HAN suspended at 0.1% in 0.1% Triton X-190 has little, if any, effect on the ticket detection limit for carbaryl.

e. Xylene: Results of interference testing with xylene, another component of some formulated pesticides, have been less lucid. It seems that xylene at concentrations less than 1% has little effect on the ability of tickets to produce color. At higher concentrations, results have been ambiguous with no clear-cut trend toward complete ticket inactivation.

2. Metal ions: Limited studies have been done during earlier programs at MRI concerning inhibition by metal ions of cholinesterase impregnated on polyurethane foam pads.[21] Some inhibition was noted for zinc sulfate and mercuric chloride but no inhibition was seen with several other metal ions at 10 ppm. However, no information existed as to the sensitivity of enzyme tickets to inhibition by metals. Since metals are found in many natural and purified water supplies, it was considered important to determine the effects of dissolved metals on the enzyme tickets.

Water quality data for rivers from seven states prepared by the United States Geological Survey were gathered and studied.[34-40] The highest concentrations of each of the metal ions found in the natural waterways were recorded. Solutions of salts of each of these ions were prepared in the laboratory at these maximal ion concentrations. The solutions were then tested for their abilities to inhibit cholinesterase test tickets.

Results of these tests are presented in Table 11. The first column of the table gives the compound used in the laboratory at the concconcentrations stated in the second column. The metal ion of interest for each trial is given in the third column and its highest reported naturally occurring concentration is presented in column four. Note that concentrations of the test compounds were adjusted to provide the maximum reported metal ion concentrations. Each solution was tested three times and the observed color intensities are presented in columns 5, 6 and 7.

Of the 18 metal ions investigated, only one, mercury(II), caused noticeable inhibition. That mercury(II) inhibits the enzyme tickets is not surprising since mercuric chloride inhibited cholinesterase in the earlier study. Inhibition by zinc was also expected but not seen. It is possible that higher concentrations of zinc could cause inhibition of the enzyme tickets.

These results indicate that, for the metals studied (except Hg^{++}) at concentrations expected in natural waterways, there should be no interference with the enzyme test tickets. It is possible that the positive response (inhibition) of the tickets to mercury(II) could be used for the detection of this ion at low levels (ppb) in water.

It is conceivable that tap water could contain much higher concentrations of dissolved metals than those tested here, which could cause ticket

TABLE 11

METAL INTERFERENCE OF EEL CHOLINESTERASE TICKETS[a/]

Compound	Test Compound Concentration (mg/ℓ)	Metal Ion	Maximum Reported Metal Ion Concentration (µg/ℓ)[b/]	Test 1	Test 2	Test 3
H_2O (control)	-	-	-	+3	+3	+3
$Na_2B_4O_7 \cdot 10H_2O$	40.564	B^{+++}	4,600	+3	+3	+3
$Na_2HAsO_4 \cdot 7H_2O$	0.133	As^{+5}	32	+3	+3	+3
$NaAsO_2$	0.055	As^{+++}	32	+3	+3	+3
$FeCl_2 \cdot 4H_2O$	4.272	Fe^{++}	1,200	+3	+3	+3
$FeCl_3 \cdot 6H_2O$	5.808	Fe^{+++}	1,200	+3	+3	+3
$K_2Cr_2O_7$	0.087	Cr^{+6}	31	+3	+3	+3
KCl	0.05721	K^{+}	30	+3	+3	+3
HgI_2	0.00113	Hg^{++}	0.5	+1.5	+1.5	+1.5
$MgCl_2 \cdot 6H_2O$	11.706	Mg^{++}	1,400	+3	+3	+3
$CoCl_2 \cdot 6H_2O$	0.044	Co^{++}	10	+3	+3	+3
$NiCl_2 \cdot 6H_2O$	0.251	Ni^{++}	62	+3	+3	+3
$CaCl_2$	0.238	Ca^{++}	86	+3	+3	+3
$ZnCl_2$	2.919	Zn^{++}	1,400	+4	+3	+3
$MnCl_2 \cdot 4H_2O$	0.865	Mn^{++}	240	+3	+4	+3
$BaCl_2 \cdot 2H_2O$	1.423	Ba^{++}	800	+3	+4	+3
$CuSO_4 \cdot 5H_2O$	0.111	Cu^{++}	25	+3	+3	+3
$AgNO_3$	0.315	Ag^{+}	200	+3	+3	+3
$Pb(C_2H_3O_2)_2 \cdot 3H_2O$	0.084	Pb^{++}	46	+4	+3	+3

a/ Tickets were tested for color production by dipping in deionized water for 15 sec (ticket-wetting step), incubating for 15 min (enzyme-inhibitor reaction step), and pressing between thumb and forefinger for 2 min (color development step).

b/ The highest concentration of metal ions found in natural waterways of seven states as reported by the United States Geological Survey, 1973-1978.

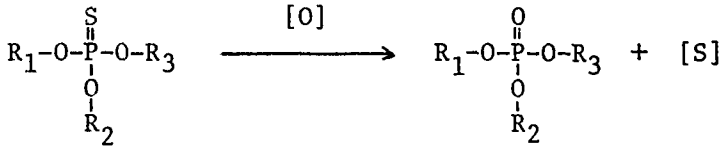
inhibition leading to false positive results. We suggest that if this is suspected in the field, the operator should test the tap water for inhibition before it is used in pesticide cleanup operations.

D. Enhanced Ticket Sensitivity with Bromine Oxidation

The enzyme ticket detection limits for diazinon, Dursban®, and malathion reported in Section V-A-4 were all 100 ppm or greater. It was important that the enzyme ticket or the test procedure be modified in some way to decrease the detection limits for these pesticides. Several methods designed to increase sensitivity of the tickets were considered and some were tested, including increasing the incubation temperature of the enzyme disc after exposure to pesticide, increasing the enzyme-inhibitor incubation time, and impregnating the enzyme discs with lower amounts of cholinesterase. Each of these procedures, however, unfavorably affected the utility or the storability of the enzyme test tickets.

1. Theory of bromine oxidation: A subset of the so-called organophosphate insecticides are more properly identified as phosphorothionates since they have a sulfur atom rather than an oxygen atom double-bonded to the phosphorus atom. It has been noted that the oxygen analogues, or phosphates, of these phosphorothionates are better inhibitors of cholinesterase than are the thionates themselves. Interestingly, the three "organophosphates" of interest in this program (diazinon, Dursban®, and malathion) are actually phosphorothionates. (Malathion is technically a phosphorothiolothionate with an additional oxygen atom replaced by a sulfur atom.) It was reasoned that greater ticket sensitivity toward these three compounds might be available by converting them to true organophosphates. Fortuitously, these are the three insecticides whose detection limits with the enzyme tickets should be improved.

The chemistry of this conversion has been termed "oxidative desulfuration" and has been claimed to occur using bromine gas on TLC plates.[8,32]



Phosphorothionate Phosphate

It was considered possible that such a reaction could be induced in solution using bromine water.

2. Detection limits after bromine treatment: Formulated pesticides were diluted with water to give the desired concentrations. A 20-ml aliquot of pesticide solution was either treated with 10 µl of 1% (0.19 M) bromine in water for 3 min, or left untreated. These solutions were then tested with eel cholinesterase tickets (1.2 units/disc) prepared according to the procedures described in the Appendix.

The enzyme discs of the test tickets were dipped for 15 sec in the solution to be tested, incubated for 15 min, and then pressed together with the attached substrate discs for 2 min. Color intensities were estimated using the Munsell Color Chart; tests were done in replicates of five. Results were averaged and plotted with standard deviations. Detection limits were taken as that concentration of pesticide that gave complete inhibition of the enzyme ticket in five of five trials.

Results are summarized in Table 12. It is apparent that while bromine pretreatment of solutions of Baygon® and carbaryl had no effect on their detection limits, bromine treatment markedly enhanced ticket sensitivities to the phosphorothionates, diazinon, Dursban®, and malathion. After treatment with bromine, all of the pesticides are detectable at levels that are sufficiently low to signal early breakthrough from charcoal columns.

TABLE 12

ENZYME TICKET DETECTION LIMITS WITH AND WITHOUT BROMINE TREATMENT

Pesticide[b]	Detection Limit (ppm, w/v)[a]	
	No Bromine[c]	With Bromine[d]
Baygon®	2	2
Carbaryl	9	9
Diazinon	300	5
Dursban®	100	0.5
Malathion	75	0.5

a/ Detection limit is the lowest concentration of pesticide that gives complete inhibition of enzyme impregnated tickets. Eel cholinesterase tickets (1.2 units/disc) were prepared according to procedures given in the Appendix.

b/ Formulated pesticides were diluted with demineralized water to give desired concentrations.

c/ Untreated 20-ml aliquots of pesticide solutions were tested with enzyme tickets.

d/ 20-ml aliquots of pesticide solutions were treated with 10 µl of 1% (v/v) (0.19 M) bromine in water for 3 min prior to testing with enzyme tickets.

The reasons for the differences between Tables 9 and 12 for the detection limits of diazinon, Dursban®, and malathion without bromine treatment are unknown (300, 100, 75 ppm versus 400, 300, 100 ppm, respectively). The differences noted for diazinon and malathion are probably within the "noise" level for detection limits determination, a measurement whose accuracy is dependent in part on the number of tests in a series of pesticide solutions. For example, an approximate determination can be made by testing a series of 200, 400, and 600 ppm pesticide solutions for inhibition. A more accurate number may be obtained by testing 200, 250, 300, 350 . . . 600 ppm solutions.

VI. STORAGE TESTING

To enhance the utility for the enzyme test tickets it was important to know how well they could withstand storage, both at elevated room temperature and under refrigeration. Additionally, since bromine treatment was so successful in decreasing detection limits, limited testing of storability of bromine water was accomplished.

A. Long-Term Storage Testing - Enzyme Tickets

The cholinesterase impregnated tickets tested under this program are to be used in Army pesticide filtration facilities as positive or negative tests for the presence of carbamate and organophosphate pesticides in water. As currently envisioned, the tests will be performed regularly by the plant monitor on water samples obtained from the water filtration stream. According to this operating scenario, it will be important to know the storability of the tickets under various conditions. Thus, at the beginning of this study, batches of tickets were prepared according to procedures given in the Appendix. One batch was made with electric eel acetylcholinesterase and another with horse serum cholinesterase. The tickets were tested to verify their abilities to form color of $\geq$ +3 intensities in the absence of pesticides. One half of each batch was put in storage at 4°C and the other half was stored at 40°C. At approximately monthly intervals, 10 tickets from each of the four experimental conditions were withdrawn from storage and tested for color formation using water instead of a pesticide sample.

Results for storage of tickets made with horse serum cholinesterase are shown in Figure 5. It is clear that horse serum tickets stored at 40°C begin to fail soon after initiation of storage. Horse serum discs stored at 4°C, however, retain their abilities to form +3 colors even after 8 months.

Tickets made with eel cholinesterase withstood the 40°C storage much better. Results in Figure 6 show no downward trend in average color intensities for tickets stored at either 4°C or 40°C for 8 months.

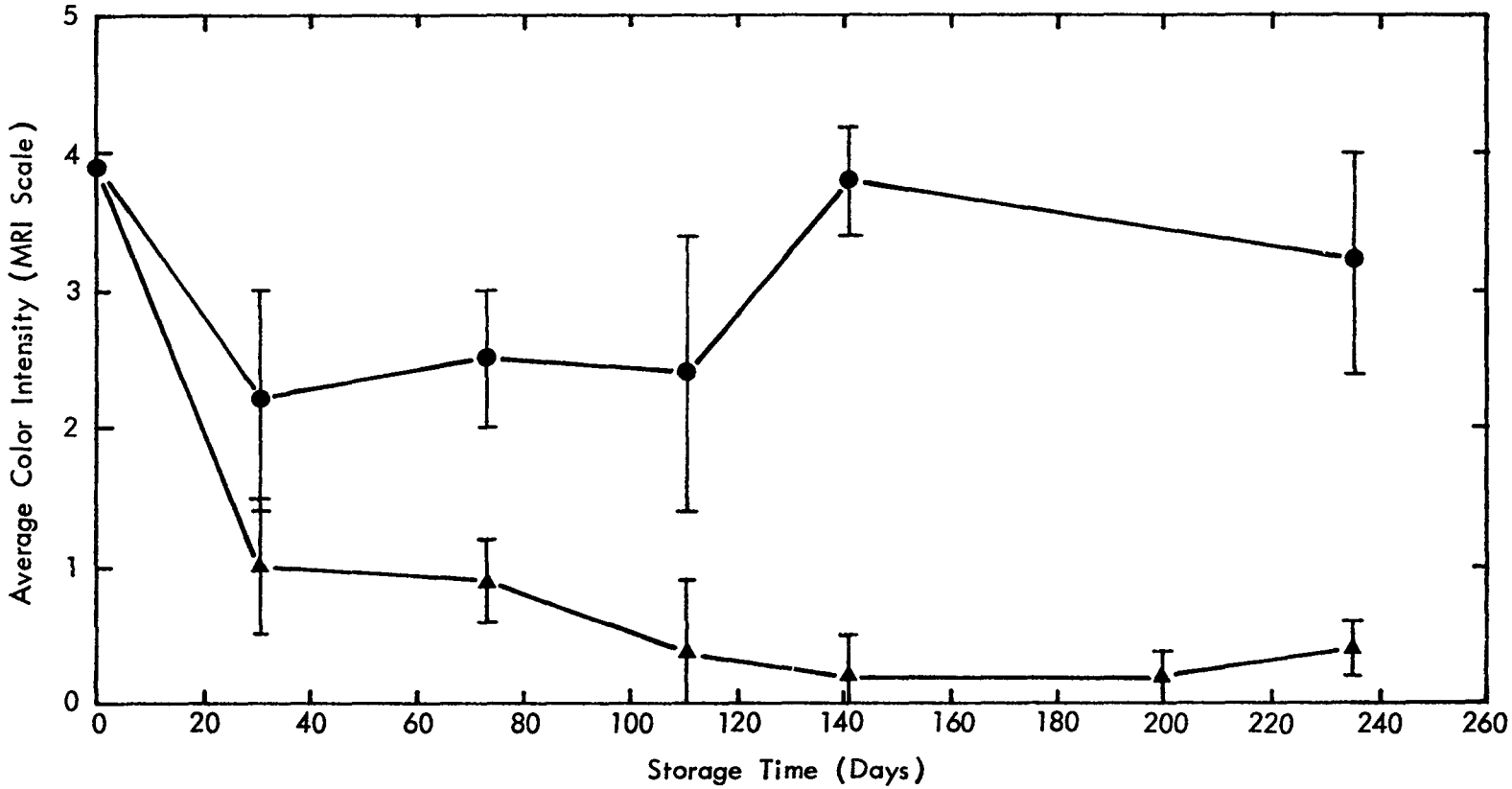


Figure 5 - Color Production With Horse Serum Cholinesterase Tickets (0.6 unit/disc) Stored at 4°C (circles) or 40°C (triangles). Points represent average color intensity observed with 10 trials. Vertical lines show ± one standard deviation.

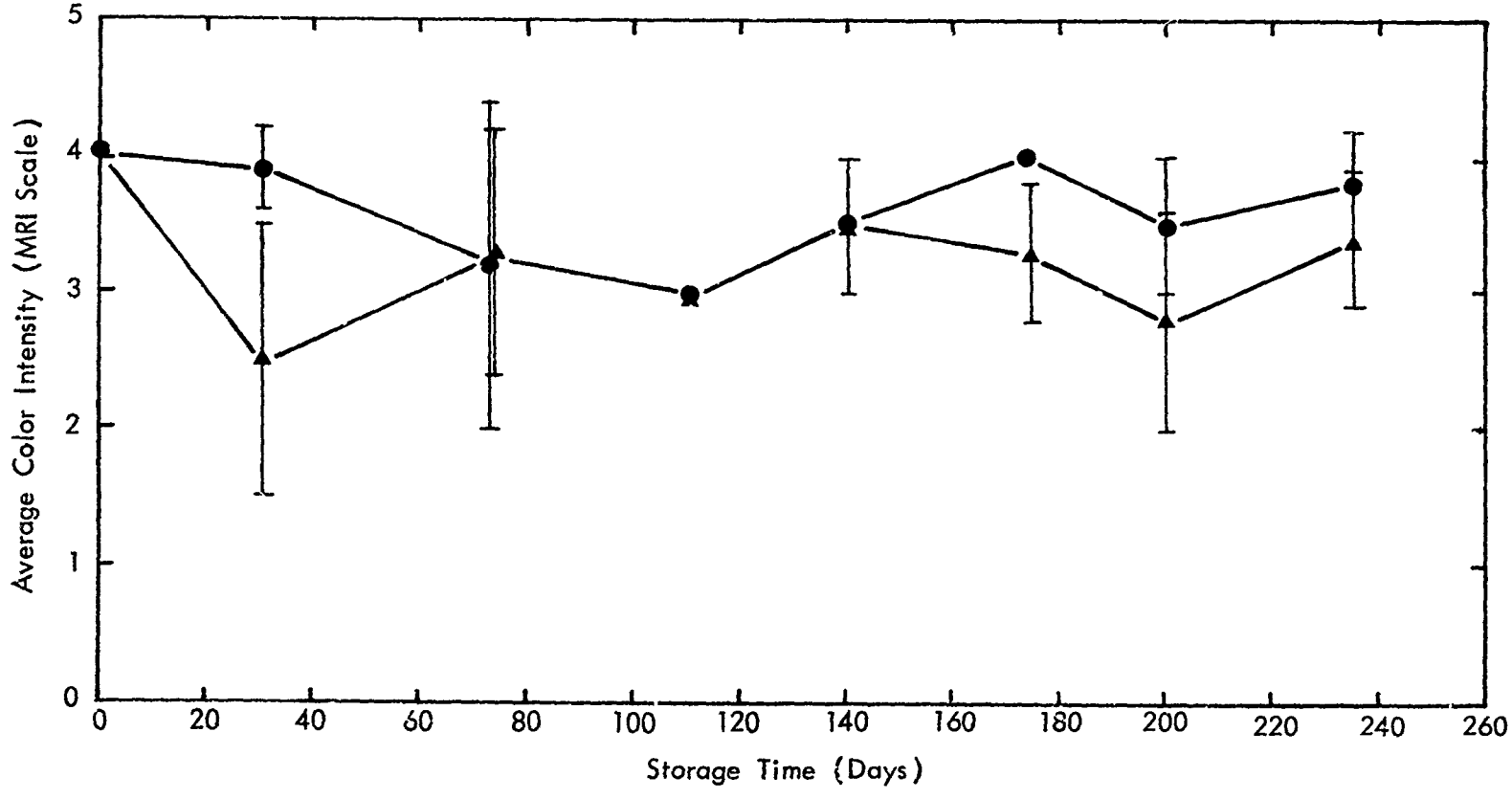


Figure 6 - Color Production With Eel Cholinesterase Tickets (1.2 units/disc) Stored at 4°C (circles) or 40°C (triangles). Points represent average color intensity observed with 10 trials. Vertical lines show ± one standard deviation.

Eel cholinesterase tickets that had been stored at 4°C or 40°C for 8 months were also tested for inhibition by 2 ppm Baygon® in water. This concentration of Baygon® was determined to be the lower limit for detection as reported in Section V-A-4. Ten replicate tickets from each storage temperature were completely inhibited by the pesticide solution.

These results indicate that eel cholinesterase tickets, when prepared according to the procedures given in the Appendix, retain their enzyme activities and pesticide detection limits for at least 8 months storage at 4°C (39°F) or 40°C (104°F).

B. Storage Stability - Bromine Water

Stability of the bromine solution is of critical importance in maintaining the appropriate bromine concentration. For field use, bromine water must be stored for long periods of time. Evaporation of the solution, dissolution in a plastic container, or decomposition [$3Br_2(aq) + 3H_2O \rightleftarrows BrO_3^- + 5Br^- + 6H^+$] may all cause changes in the bromine concentration. To test these possibilities, four bromine solutions at 0.25% were prepared. Two were made in demineralized water. One of these was stored in a small glass bottle while the other was stored in a polyethylene plastic bottle. The other two bromine solutions were made in 0.12 M HCl. It was reasoned that free protons would stabilize Br_2 according to the above reaction. One of these solutions was stored in an identical glass bottle while the other was stored in an identical plastic bottle.

Periodically, one drop of each of the four bromine solutions was added to 20 ml of either water or various concentrations (1, 5, 10 ppm) of formulated diazinon in water. The 20-ml solutions were then tested for their abilities to inhibit the enzyme on cholinesterase test tickets.

Within 1 day, the bromine water stored in the plastic containers was beginning to loose its ability to enhance ticket sensitivity to diazinon. After 11 days, the bromine stored in plastic had no apparent effect on the enzyme ticket test for diazinon. Since the walls and bottoms of the plastic containers gradually took on a yellow color during this period, it is likely that bromine was being lost by dissolution into the plastic.

Bromine water stored in glass screw-capped bottles maintained its oxidizing power much better. After 31 days, bromine stored in glass was still capable of rendering 5-ppm diazinon solutions capable of completely inhibiting enzyme tickets. This ability, however, seemed to slowly degrade over the next 20 days. At that time, bromine-treated solutions of 5 ppm diazinon gave +1.5 intensities upon testing with enzyme tickets rather than no color. Results with bromine in 0.12 M HCl were not significantly different. This slow decay of oxidation ability is believed to be due to evaporation of the bromine from

the bottle during frequent withdrawal of aliquots for testing. Bromine water stored in sealed glass ampoules or single use capillary tubes will last much longer.

VII. ENZYME TICKET DELIVERY TO USAMBRDL

Ten prototype pesticide test kits were prepared by MRI for transferral to the U.S. Army Medical Research and Development Command. It is anticipated that they will be tested under field conditions at prototype pesticide filtration facilities.

A. Pesticide Test Kit Description

Each test kit is contained in a case that is approximately 23 cm wide by 14 cm deep by 14 cm tall with a carrying handle mounted on the hinged top. Inside the lid is a 20 cm by 12 cm card containing simple operating instructions for the kit (see Section VII-B). On hundred enzyme test tickets prepared and packaged according to the procedures given in the Appendix are stored in two 12 x 6 cm compartments. A third compartment contains a 50-ml beaker and 100 sealed capillary tubes each containing enough bromine water for testing one 20-ml sample of pesticide solution. The bromine tubes have an inside diameter of 1.2 mm and are about 3 cm long. Each sealed tube contains 20 µl of 0.5% (0.085 M) bromine in water.

B. Test Procedure

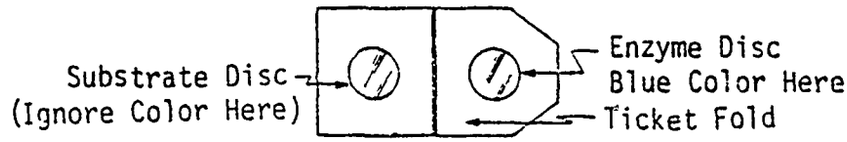
The following information is given on the kit instruction card.

Contents: 100 foil-packaged tickets, 100 yellow bromine tubes (4 packets of 25 tubes), 1 glass beaker, 2 glass rods (1 extra)

Test Procedure: (Read entire procedure before starting)

1. Remove beaker from case and add sample water to "-20" mark.
2. Place beaker on flat surface.
3. Remove 1 packet of bromine tubes and tear along black line.
4. Slide cardboard holder out to remove 1 bromine tube. IF LIQUID IN TUBE IS COLORLESS, DO NOT USE.
5. Place tube in beaker and, using glass rod, crush tube against bottom of beaker until yellow color is no longer visible.
6. Wait 3 minutes.
7. While waiting, remove 1 packaged ticket from case. Tear notched end of packet, remove contents and discard white cardboard.

8. After the 3 minutes, fold back loose section of overlay and wet exposed enzyme disc (paper disc at end of ticket with clipped corners) by dipping in beaker for 15 seconds.
9. Return ticket to packet and set aside for 15 minutes.
10. After 15 minutes, peel off and discard overlay, exposing the substrate disc.
11. Fold ticket so the 2 discs make contact and hold for 2 minutes.
12. After 2 minutes, open the ticket and observe the color of the enzyme disc.



Enzyme Ticket

Results:

If disc is any shade of blue, test is negative. Pesticide level is below reported detection limits.

If disc remains white, test is positive. Pesticide may be present. However, a control test must be run to verify performance of tickets.

Control Test:

Rinse beaker thoroughly with clean water, take new ticket and proceed as in steps 6 through 12 using drinking water in place of sample water.

If control disc is blue, tickets are good and presence of pesticide in original sample water is verified.

If control disc is white, remaining tickets may be bad.

REFERENCES

1. Standard Methods for the Examination of Water and Wastewater, A. E. Greenberg (APHA), and M. J. Taras (AWWA), Eds., American Public Heath Association, American Water Works Association and Water Pollution Control Federation, publishers, 1976.

2. Slade, P., "IUPAC Commission on the Development, Improvement, and Standardization of Methods of Pesticide Residue Analysis," J. of the AOAC, 59, 894-910 (1976).

3. Oller, W. L., and M. F. Cranmer, "Analysis of Chlorinated Insecticides and Congeners," J. Chromatographic Sciences, 13, 296-300 (1975).

4. Debbrecht, F. J., and E. M. Neel, "A Completely Portable Gas Chromatograph for On-Site Analysis," Am. Lab., 3, 39-44 (1971).

5. Luckens, M. M., "A Rapid Procedure for the Laboratory Diagnosis of Poisoning by Organochlorine Insecticides," from Pesticide Symposia, collected papers from the Inter-American Conference on Toxicology and Occupational Medicine, 6th and 7th, 1968-1970.

6. Mendoza, C. E., and J. B. Shields, "Determination of Some Carbamates by Enzyme Inhibition Techniques Using Thin-Layer Chromatography and Colorimetry," J. Agr. Food Chem., 21, 178-184 (1973).

7. Mendoza, C. E., "Enzyme Inhibition and Chromatographic Techniques: Comparative Studies and Application to Pesticide Residue Analysis," Intern. J. Environ. Anal. Chem., 3, 171-183 (1974).

8. Mendoza, C. E., "Analysis of Pesticides by the Thin-Layer Chromatographic Enzyme Inhibition Technique, Part II," Residue Review, 50, 43-72 (1974).

9. Burchfield, H. P., and E. E. Storrs, "Analysis of Organophosphorus Insecticides and Metabolites," J. Chrom. Sci., 13, 202-211 (1975).

10. Dorough, H. W., and J. H. Thorstenson, "Analysis of Carbamate Insecticides and Metabolites," J. Chrom. Sci., 13, 212-224 (1975).

11. Frode, H. C., and I. B. Wilson, "Acetylcholinesterase," The Enzymes, Vol. II, 3rd Ed., P. D. Boyer ed., Academic Press, New York, 87-114, 1971.

12. Rosenberg, T. L., "Acetylcholinesterase," Adv. in Enzymology, 43, 103-218 (1975).

13. Aldridge, W. N., and E. Reiner, eds., "Enzyme Inhibitors as Substrates, Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids," in Frontiers of Biology, 26, American Elsevier, New York, 1972.

14. Guilbault, G. C., Handbook of Enzymatic Methods of Analysis, Marcel Dekker, Inc., New York, New York, 1976.

15. Bauman. E. K., L. H. Goodson, G. G. Guilbault, and D. N. Kramer, "Preparation of Immobilized Cholinesterase for Use in Analytical Chemistry," Anal. Chem., 37, 1378-1381 (1965).

16. Bauman, E. K., L. H. Goodson, and J. R. Thomson, "Stabilization of Serum Cholinesterase in Dried Starch Gel," Anal. Biochem., 19 (3), 587 (1967).

17. Goodson, L. H., and W. B. Jacobs, "A Rapid Detection System for Organophosphates in Water," Proceedings 1972 National Conference on Control of Hazardous Material Spills, Environmental Protection Agency, University of Houston, Houston, Texas, March 21-23, 129-136, 1972.

18. Goodson, L. H., and W. B. Jaccbs, "Rapid Detection System for Organophosphates and Carbamate Insecticides in Water," Environmental Protection Technology Series, EPA-R2-72-010, August 1972.

19. Goodson, L. H., W. B. Jacobs, and A. W. Davis, "An Immobilized Cholinesterase Product for Use in the Rapid Detection of Enzyme Inhibitors in Air or Water," Anal. Biochem., 51, 362-367 (1973).

20. Goodson, L. H., and W. B. Jacobs, "Application of Immobilized Enzymes to Detection and Monitoring Problems," 1973 Engineering Foundation Conference on Enzyme Engineering, Henniker, N. H., August 5-10, 1973, in Enzyme Engineering, Vol. 2, E. K. Pye and L. B. Wingard, Jr. (Eds.), Plenum Publishing Company, New York (1974).

21. Goodson, L. H., and W. B. Jacobs, "Use of Immobilized Enzyme Products in Water Monitoring," Proceedings of the 1974 National Conference on Control of Hazardous Material Spills, American Institute of Chemical Engineers, New York (1974).

24. Goodson, L. H., and W. B. Jacobs, "Recently Developed Enzymatic System for Environmental Monitoring," Proceedings of 6th Annual Symposium of American Defense Preparedness Association, April 29, 1975, Edgewood Aresenal Special Publication EO-SP-76001, January 1976.

25. Goodson, L. H., and W. B. Jacobs, "Evaluation of CAM-1, A Warning Device for Hazardous Material Spills," Contract No. 68-03-0299, Task I Report, Environmental Protection Technology Series, EPA-600/2-77-219, November 1977.

26. Goodson, L. H., and W. B. Jacobs, "Solution Sampling Enzyme Alarm Studies," Special Technical Report, March 1975, Edgewood Arsenal Contractor Report ED-CR-75006, 76 pages.

27. Goodson, L. H., and W. B. Jacobs, "Real Time Monitor, Immobilized Enzyme Alarm and Spare Parts," Final Comprehensive Report, February 1976, NTIS No. AD-A036,372, Edgewood Arsenal Contractor Report ED-CR-77015, 109 pages.

28. Halpern, B. D., S. L. Ledis, and G. G. Gardner, "Studies on Eel Cholinesterase for Use in Detection Devices," Final Report, March 1977, NTIS No. ad-A039822, Edgewood Arsenal Contractor Report ED-CR-76044, 70 pages.

29. Goodman, A., G. Smith, R. M. Gamson, "The Effect of Organophosphorus Insecticides on Nerve Agent Detector Tickets," May 1975, Edgewood Arsenal Technical Report, ED-TR-75008, 16 pages.

30. Goodson, L. H., and M. Rogers, "Detector Kit for Chemial Agents in Water," Final Comprehensive Report, June 1977, Edgewood Arsenal Contractor Report ARCSL-CR-77025, 47 pages.

31. Goodson, L. H., and D. R. Sellers, "Feasibility Studies on Enzyme Systems for Detector Kits," Draft Quarterly Progress Report No. 9, December 1978, Edgewood Arsenal Contractor Report No. ARCSL-CR-79023, 45 pages.

32. Eto, M., Organophosphorus Pesticides: Organic and Biological Chemistry, CRC Press, Cleveland, 1974.

33. Ellman, G. L., K. D. Courtney, V. Andres, Jr., and R. M. Featherstone, "A New Rapid Colorimetric Determination of Acetylcholinesterase Activity," Biochem. Pharmacol., 7, 88-95 (1961).

34. Water Resources Data for Missouri, United States Department of Interior, U.S. Geographical Survey, Rolla, Missouri, USGS, 1973.

35. Water Resources Data for New York - water year 1975. Water Data Report NY-75-1, USGS, 1976.

36. Water Resources Data for North Carolina - water year 1975. Water Data Report NC-75-1, USGS, 1976.

37. Water Resources Data for Arizona - water year 1977. Water Data Report AZ-77-1, USGS, 1978.

38. Water Resources Data for California - water year 1977. Vol. 4. Water Data Report CA-77-4, USGS, 1978.

39. Water Resources Data for Florida - water year 1977. Vol. 2A. Water Data Report FL-77-2A, USGS, 1978.

40. Water Resources Data for Texas - water year 1977. Vol. 3. Water Data Report TX-77-3, USGS, 1978.

APPENDIX

MATERIALS AND METHODS FOR ENZYME TICKET FABRICATION

MATERIALS AND METHODS FOR ENZYME TICKET FABRICATION

I. EEL CHOLINESTERASE DISCS

A. Buffer Solution (40 ml, 0.5 M, pH 8.0)

1. To prepare 0.5 M PIPPS (1,4-bis(3-sulfopropyl)piperazine) buffer solution, suspend 6.00 g (M.W. 330) in 30 ml of deionized water.

2. Adjust the pH to 8.0 using 5 M NaOH.

3. Add 0.20 g bovine serum albumin (Fraction V, Sigma Chemical Company) (0.5%).

4. Add 0.6 ml 1.0% Triton X-100 solution (Sigma Chemical Company) (0.015%).

5. Dilute to 40 ml with deionized water.

B. Enzyme Solution (for use in making discs with 1.2 units/disc)

1. Dissolve ∿ 10 mg eel cholinesterase (Code ECH, Worthington Biochemical Company, ∿ 100 units/mg) in 30 ml of the above PIPPS buffer solution.

2. Refrigerate at 5°C for 2 to 3 hr, but not less than 2 hr, prior to analyis on pH Stat. (Note: The activity of the enzyme in solution changes with the age of the solution.)

C. Enzyme Solution Analysis on pH Stat

1. To prepare 1 liter of substrate solution, dissolve the following in about 900 ml of pH 5.5 deionized water:

8.120 g magnesium chloride ($MgCl_2 \cdot 6H_2O$) (0.04 M).
5.850 g sodium chloride (NaCl) (0.10 M).
0.730 g acetylcholine chloride (0.004 M).

Dilute to 1 liter with pH 5.5 deionized water.

Note: Solution is stable for 10 days if kept refrigerated at 5°C.

2. Operating procedure for Sargent pH stat:

a. Turn on pH stat and allow to warm up (about 15 min).

b. Adjust temperature controller to 37°C.

c. Set instrument to pH 8.0.

d. Calibrate instrument using about 50 ml stirred, preheated (37°C) 0.1 M borate buffer pH 8.0 and adjust the recording pen for minimum movement.

e. Fill the 2.5 ml delivery burette with standardized 0.02 M NaOH.

3. Analysis of enzyme solution.

a. Measure exactly 50.0 ml of substrate solution into a 100-ml ground-glass-bottom beaker. (Note: A flat-bottom beaker is necessary to assure good heat transfer from the thermoelectric cooler of the pH stat.)

b. Preincubate substrate solution in 37°C water bath for about 10 min. (This can be done while pH stat is warming up.)

c. Place the ground-glass beaker on the pH stat temperature controller and allow to equilibrate to 37°C (two cycles).

d. Add 0.100 ml enzyme solution to stirring substrate solution and initiate titration.

e. Add pH 8.0, 0.02 M NaOH by dropwise addition until recorder shows change in baseline.

f. Record linear progression of analysis for about 5 to 10 min.

g. Calculate enzyme activity from linear slope.

4. Enzyme activity calculation (units/mg): Average of three titrations is used for final activity.

$$\text{units/mg} = \frac{(\text{ml NaOH/min})\ (\text{M NaOH} \times 10^3)}{(\text{enzyme conc. (mg/ml)}) \times (\text{ml of enzyme solution added})}$$

D. Enzyme Solution Application to Discs

The units of enzyme desired on each disc are to be contained in 30 µl (i.e., the volume of enzyme solution to be added to each 1/2 in. paper circle).

1. Calculate the total number of units of enzyme in the enzyme solution:

Total units of enzyme = (mg of enzyme used to make solution) x (assayed activity (units/mg) of enzyme product).

2. Calculate the units of enzyme contained in 30 µl of solution:

$$\text{units} = \frac{(\text{Total units of enzyme})\ (0.030\ \text{ml})}{(\text{Volume of solution (ml)})}$$

If units/30 µl is significantly greater than desired units/disc, the solution may be diluted with PIPPS buffer to a new "adjusted volume."

3. Calculation of "adjusted volume."

$$\text{Adjusted volume} = \frac{(\text{units}/30\ \mu\text{l, found})\ (\text{vol. of enzyme solution})}{(\text{units}/30\ \mu\text{l, desired})}$$

If the adjusted volume is greater than the original volume of the enzyme, it may be diluted with the original buffer solution to the "adjusted volume." If the enzyme solution is already too dilute, slightly more enzyme solution (i.e., up to 40 µl/disc) may be added, but this practice is to be avoided whenever possible.

E. Enzyme Disc Preparation

1. Arrange 1/2 in. Whatman No. 1 paper discs (30 ml of enzyme solution will make about 1,000 discs) on thoroughly cleaned glass plates.

2. Pipet 30 µl of enzyme solution on each disc using a Pipetman pipet.

3. Allow to air-dry for 2 to 3 hr at room temperature.

4. Place discs in uncapped glass vials and allow to dry 5 to 20 hr at $\leq$ 0.01 mm Hg, at 25°C in a vacuum desiccator before storage or packaging. Use clean desiccator (i.e., without indoxyl acetate vapors).

5. Admit dried air before removing desiccator lid; seal containers tightly and refrigerate at 5°C inside a second container with a desiccant.

II. INDOXYL ACETATE DISCS (for use with eel cholinesterase discs)

A. Solution for Substrate Discs

In 100 ml deionized water, pH 5.5, dissolve both 0.0448 g potassium ferricyanide ($K_3Fe(CN)_6$) (Mallinckrodt Chemical Company) and 0.0715 g potassium ferrocyanide ($K_4Fe(CN_6)\cdot 3H_2O$) (Baker Chemical Company).

B. Applying Solutions to Paper Discs

1. Arrange 9.0 cm Whatman No. 1 filter paper circles on clean glass plates making sure that none overlap or touch.

2. Pipet ferri/ferro solution (1.0 ml/circle) and allow to air-dry for 2 to 3 hr.

Note: Protect substrate solutions and discs from exposure to light during all further handling procedures.

3. In a separate covered beaker, dissolve indoxyl acetate (18 mg/ml) in A.R. acetone.

4. Pipet indoxyl acetate solution (1.0 ml/circle) evenly over the entire surface area of the filter paper circles and allow to air-dry about 30 min before punching into 1/2 in. discs.

C. Vacuum Desiccator Drying

Use separate desiccators for enzyme and substrate discs. Follow same instructions for enzyme disc drying, but protect substrate from light. Store indoxyl acetate discs in tightly sealed containers inside desiccated containers inside a refrigerator.

III. HORSE SERUM CHOLINESTERASE DISCS

A. Buffer Solution (40 ml, 0.5 M, pH 8.0)

1. To prepare 0.5 M PIPPS buffer solution, suspend 6.60 g (M.W. 330) in 30 ml of deionized water.

2. Adjust to pH 8.0 using 5 M NaOH.

3. Add 0.20 g bovine serum albumin (Fraction V, Sigma Chemical Company) (0.5%).

4. Add 1.00 ml 1% Aerosol OF (w/v) (Sigma Chemical Company) (0.025%).

5. Dilute to 40 ml with deionized water.

B. Enzyme Solution (for use in making discs with 0.6 units/disc)

1. Dissolve about 85 mg horse serum cholinesterase (Sigma Chemical Company, ∿ 15 units/mg) in 30 ml of the above PIPPS buffer solution.

2. Refrigerate at 5°C for exactly 2 hr prior to analysis on pH stat. (Note: Activity of the enzyme solution changes with time.)

3. Proceed as for eel cholinesterase disc preparation.

IV. 2,6-DICHLOROINDOPHENYL ACETATE SUBSTRATE DISCS (DCIPA; for use with horse serum cholinesterase discs)

A. Solution for Substrate Discs

Dissolve the DCIPA (5.0 mg/ml) in A.R. acetone.

Note: Keep beaker covered to minimize evaporation while stirring and protect from light.

B. Applying Solutions to Substrate Discs

1. Arrange 9.0 cm Whatman No. 1 filter paper circles on clean glass plates making sure that none overlap or touch.

2. Pipet (1 ml/circle) DCIPA solution (5.0 mg/ml) and allow to air-dry about 1 hr under subdued light.

3. When dry, cut circles into 1/2 in. discs using a clean steel punch.

4. Place substrate discs in glass containers and vacuum dry following the same procedure for enzyme discs.

C. Packaging and Storage of DCIPA Substrate Discs

Same procedure as for indoxyl acetate discs. Enzyme and substrate discs should not be stored in same container.

V. ENZYME TICKET FABRICATION

1. Wash the 0.020 in. polyethylene (after it has been cut into the shape of tickets 2-1/2 in. by 1-1/4 in. with corners clipped from one end) sheet in hot Alconox solution in an ultrasonic cleaning bath for 30 min, rinse in deionized water, blot excess water with paper towels, and dry in an oven for 15 min at 90°C. Degas these polyethylene pieces in a desiccator at 0.01 mm Hg or less for 18 hr.

2. Heat-seal one enzyme disc to the end of each polyethylene support with the clipped corners.

3. Heat-seal a substrate disc to the other end of each support.

4. Complete ticket fabrication by heat-sealing a cover of laminated plastic foil (Apollo) over the substrate disc.

5. Place the completed enzyme ticket in a bag 2 in. by 3-1/4 in. made from the laminated foil packaging material (Apollo) along with a 1-1/4 in. by 2-1/2 in. piece of Proteksorb® silica gel paper which has been freshly activated by heating to 150°C for 1 hr. Heat-seal the bag and store in refrigerator.

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