A METHOD FOR THE VISUAL ESTIMATION OF ERYTHROCYTE CHOLINESTERASE ACTIVITY

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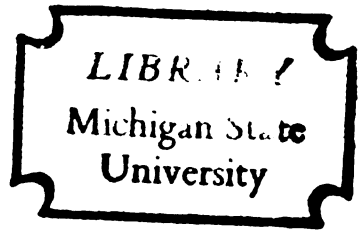
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ABSTRACT

A METHOD FOR THE VISUAL ESTIMATION OF
ERYTHROCYTE CHOLINESTERASE ACTIVITY

by Louise K. Mueller

Reagent and standard impregnated strips were used in a screening procedure for the visual estimation of erythrocyte cholinesterase activity in samples of whole blood. Thiocholine released by enzyme action on acetylthiocholine substrate reacts quantitatively with dithiobisnitrobenzoic acid to yield a yellow anion. The time required for the production of a standard amount of yellow anion was used as a measure of enzyme concentration. The action of plasma esterase was inhibited by quinidine sulfate.

Test reagent strips were sufficiently stable to be used 3.5 months. Standard reagent strips were stable at least one month. The precision of visual estimation of enzyme activity was not affected by varying the intensity of the standard color or the rate of approach to a standard color. Correction factors relating rate of enzyme reaction at temperatures ranging from 15-35 C. to a base temperature of 25 C. were calculated. The time of color change was related to temperature and level of erythrocyte cholinesterase activity at the mean activity of the normal samples and 71% mean activity.

A decrease in erythrocyte cholinesterase activity of 45% or more could be detected with a 5.8% index of discrimination. The method had a precision of $\pm$ 25%.

A METHOD FOR THE VISUAL ESTIMATION OF ERYTHROCYTE CHOLINESTERASE ACTIVITY

By

Louise K. Mueller

A THESIS

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For Bill

Jim, Cathy, and Dave

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	3
Location of the Enzyme	5
Transmission of Nerve Impulses	6
Mechanism of Transmission of Nerve Impulses	6
Effects of Decreased Cholinesterase	7
Measurement of Cholinesterase Activity	8
MATERIALS AND METHODS	11
Preparation of Test and Standard Reagent Strips	12
Visual Quantitation of Enzyme Activity	12
Reference Method	13
Modified Visual Method	13
Temperature-Rate Studies	14
RESULTS	15
DISCUSSION	21
SUMMARY AND CONCLUSIONS	24
REFERENCES	26
APPENDICES	30
VITA	33

LIST OF TABLES

Table		Page
1	Comparison of reagent absorbances using fresh reagents and lyophilized strips.	17
2	Comparison of estimates of enzyme activity (in minutes) using 3 and 5 minute color standards and a 3 minute color standard with one-half enzyme concentration	18
3	Comparison of reference method values with modified visual method values	19

LIST OF FIGURES

Figure		Page
1	Time of color change in relation to temperature and level of erythrocyte cholinesterase activity at mean activity of normal sample and 71% mean activity	20

LIST OF APPENDICES

Appendix		Page
I	Reagents	30
II	Data for Index of Discrimination	32

INTRODUCTION

In recent years there has been a sharp increase in the use of the organophosphate insecticides by agriculturalists. The basis of success for these compounds resides in their ability to combine with and non-competitively inhibit the action of cholinesterase, an enzyme involved in the transmission of nerve impulses. Unfortunately, the compounds are toxic to man as well as to insects. As a result of careless handling techniques and/or faulty equipment, the compounds enter the body through the skin or by inhalation or ingestion.

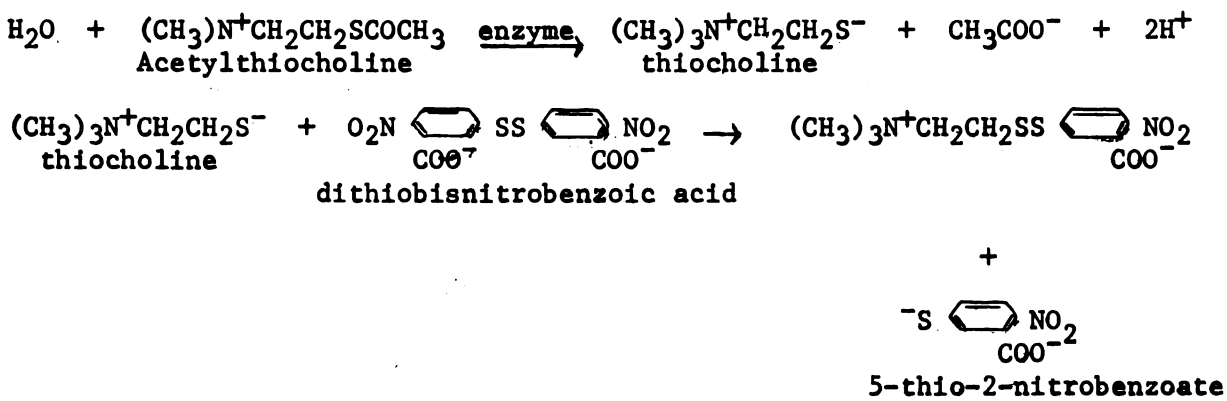
Variable decreases can occur in enzyme activity in the blood and wherever cholinoceptive membranes are located. Since the enzyme is normally present in excess, an initial exposure does not necessarily produce symptoms of poisoning other than transient symptoms at points of entry. Because of the temporary nature of such symptoms as sweating, muscular twitching, headache, and mild nausea, they are often ignored.

Repeated small exposures, however, can increase susceptibility to poisoning to a degree where serious illness characterized by rapid onset and short course occurs. Results of repeated exposures are reflected by extended periods of decreased erythrocyte cholinesterase activity. By periodically testing the blood of individuals handling organophosphates the incidence of serious illness could be reduced.

The purpose of this study was to develop for field use a rapid, simplified procedure for the visual estimation of erythrocyte

cholinesterase activity. The procedure is a modification of the spectrophotometric method of Ellman, Courtney, Andres, and Featherstone (1961). With this method erythrocyte cholinesterase activity in a whole blood sample is measured following a coupled reaction sequence.

Ellman's *et al.* (1961) equations for the reactions are as follows:



In this study, enzyme activity was estimated visually by measuring the time required to produce a standard amount of the yellow anion, 5-thio-2-nitrobenzoate. Reagents and standard were supplied in a stable, premeasured form on lyophilized reagent strips.

REVIEW OF LITERATURE

Cholinesterases are mucopolysaccharide enzymes which catalyze the hydrolysis of the choline esters of fatty acids. Mammalian species contain 2 types of the enzyme, acetylcholinesterase and butyrylcholinesterase. They differ in function, substrate specificity, reaction to inhibitors, and distribution.

Acetylcholinesterase (true cholinesterase, acetylcholine acetylhydrolase) performs the specific function of catalyzing the hydrolysis of acetylcholine, a neurohumoral transmittor substance. Following purification by column chromatography, its molecular weight, as determined from sedimentation and diffusion coefficients, is 230,000. In gel filtration studies, the enzyme moved at the same rate as catalase, suggesting a molecular weight of 250,000. Friction ratio indicates the enzyme is a globular protein. The number of active sites per molecule has been calculated as 4 (Kremzer and Wilson, 1964).

The enzyme is capable of hydrolyzing a number of choline and noncholine esters. The more closely the alcohol group in noncholine esters simulates the choline configuration, the more rapidly is the ester hydrolyzed. Expressed as a percentage of acetyl-β-methyl choline hydrolysis, the carbon analog of acetylcholine, 3:3-dimethyl butyryl acetate, is hydrolyzed most rapidly next to acetylcholine (Adams, 1949). For any given alcoholic group, the optimal acyl group for the enzyme is acetate. Chain branching at the carbon atom of the alcohol adjacent to the ester link, as in acetyl-β-methyl choline (methacholine), decreases hydrolysis

by one-third as compared to acetylcholine (Adams and Whittaker, 1949). Mendel and Rudney (1945) found that optimal acetylcholine concentration for the enzyme's activity varies with ionic strength. In the absence of salts other than 0.025 M $NaHCO_3$, optimum activity occurs at 0.00025 M acetylcholine. Following addition of 0.08 M KCl to the $NaHCO_3$, optimum activity occurs at 0.002 M acetylcholine. An absolute increase in the rate of substrate hydrolysis also occurs. The authors suggest that the escape of K^+ occurring *in vivo* upon stimulation might possibly help to maintain optimal conditions for enzyme activity. Inhibition of enzyme activity occurs at acetylcholine concentrations in excess of 10^{-3} M. Enzyme action is also pH dependent, maximum activity occurring at pH 8 to 8.5 (Wilson and Bergmann, 1950).

Butyrylcholinesterase (pseudocholinesterase, acetylcholine acylhydrolase) differs from acetylcholinesterase in that, for any given alcoholic group, the optimal acyl group is butyrate. The hydrolysis rate of butyrylcholine is twice that of acetylcholine. Benzoylcholine is also hydrolyzed, but not methacholine (Adams and Whittaker, 1949). Enzyme activity increases rapidly from pH 6 to pH 8 where it reaches maximum activity and remains until the enzyme is denatured. No inhibition of enzyme occurs in the presence of excess acetylcholine (Glick, 1937). The normal physiologic function of the enzyme is not known at present.

Butyrylcholinesterase is more sensitive to inhibition by the organophosphates, diisopropyl phosphorofluoridate (DFP) and tetraethyl pyrophosphate (TEPP) than acetylcholinesterase (Grob and Harvey, 1949). The sensitivity of both enzymes to antimalarial and related drugs has been studied by Wright and Sabine (1948). With quinidine, quinine, plasmochin, and paludrine, concentrations completely inhibiting butyrylcholinesterase had no effect on acetylcholinesterase.

Location of the Enzyme

The entire lengths of neurons giving rise to postganglionic parasympathetic, preganglionic autonomic, and somatic motor peripheral cholinergic fibers contain relatively high concentrations of acetylcholinesterase. Postganglionic sympathetic and primary afferent neurons generally have lower concentrations of the enzyme (Koelle, Davis, and Gromadzki, 1967). Levels within the CNS vary considerably. Concentration is high in neurons of the anterior and lateral horn cells and cranial motor nerves (Giacobini, 1956 and 1967).

At the skeletal muscle motor end-plates, high concentrations of acetylcholinesterase are found at the surface and invaginations of the postjunctional membrane. According to Koelle (1965), there is also an internal reserve of recently synthesized enzyme which is held within the endoplasmic membrane for replacement in the course of the cell's cycle of protein turnover.

Inhibition of this enzyme in nerve and muscle tissue produces a functional derangement in the transmission of nerve impulses.

The enzyme has also been found in erythrocyte stroma, myotendinous junctions, thrombocytes, and placental tissue (Svensmark, 1965). Function in these areas is unknown, although the enzyme's presence in erythrocytes has been proposed as a protective mechanism against nerve excitation being caused by circulatory transport of acetylcholine (Alles and Hawes, 1940).

Butyrylcholinesterase is found in the glia and white fiber tracts, the liver, pancreas, intestinal mucosa, and blood plasma. Inhibition or lack of this enzyme due to genetic variance or liver disease increases susceptibility to poisoning following administration of muscle relaxants (Koelle, 1965, and Svensmark, 1965).

Transmission of Nerve Impulses

The concept of chemical neurohumoral transmission was first advanced by DuBois-Reymond in 1877. He stated that excitation from motor-nerve terminals to effector cells occurred either electrically as a result of action currents or chemically as a result of excitor substances formed and released at the surface of the nerve endings. Although similarities were noted in response to injections of adrenal gland extracts and stimulation of the sympathetic nerves (Elliott, 1905) and injections of acetylcholine and stimulation of parasympathetic nerves (Dale, 1914), neurohumoral transmission of nerve impulses was not generally accepted.

In 1921, Otto Loewi began his classical experiments with frog hearts and established the first real proof that excitation of a nerve caused the release of a chemical substance which then transmitted the nerve impulse. The substance, named *Vagusstoff* by Loewi, was subsequently identified by Loewi and Navratil in 1926 as acetylcholine.

Final acceptance of neurohumoral transmission came with (1) the demonstration of a concentration of enzyme in nerve and motor end-plate tissue capable of hydrolyzing acetylcholine with a speed consistent with the process of transmission, (2) the localization of the enzyme at the neuronal surface where bioelectric phenomena occur, (3) the demonstration of choline acetylase, an enzyme involved in the synthesis of acetylcholine, in peripheral fibers as well as in brain, and (4) the alteration and inhibition of the nerve action potential (NAP) by anticholinesterases (Nachmansohn, 1946).

Mechanism of Transmission of Nerve Impulses

The normal resting mammalian axon has an intracellular concentration of potassium approximately 20 times that of the extracellular fluid. The

concentration of sodium is in reverse order. The maintenance of this concentration differential by sodium pump and active transport results in a transmembrane potential of approximately 70 mV, the interior of the axon negative with respect to the exterior.

The application of a stimulus above threshold level initiates a nerve action potential. Membrane permeability is altered permitting sodium ions to flow rapidly inward in response to a concentration gradient. Local reversal of membrane polarity results. Repolarization occurs immediately as permeability is decreased to sodium and increased to potassium. The polarization-repolarization process affects the adjacent resting membrane whose permeability is in turn altered. Conduction of the NAP occurs without decrement along the axon.

The arrival of the NAP at the axonal terminal causes the release of several quanta of the neurohumoral transmittor synthesized in the axon and stored in the synaptic vesicles. The transmittor substance diffuses across the synaptic cleft to receptor sites on the postjunctional membrane and alters the ionic permeability of the membrane. A generalized permeability to all ions results in an excitatory postsynaptic potential. If this exceeds threshold, an action potential is propagated in the affected nerve or muscle cell.

Destruction or dissipation by diffusion of the neurotransmittor then occurs. At cholinergic junctions, the enzyme cholinesterase assists in the destruction of the neurotransmittor by hydrolysis.

Effects of Decreased Cholinesterase

Excess acetylcholine potentiates responses of glands and muscles innervated by the parasympathetic system. Results are excessive salivation, vomiting, retching, bradycardia, and frequent micturation with incontinence.

Voluntary muscles react to excessive acetylcholine with fibrillation, fasciculations, weakness, and paralysis.

In the autonomic ganglia and in the CNS, acetylcholine has first an excitatory action and then, at higher concentrations, an inhibitory reaction. Transient hyperpnea, followed by gasping and respiratory failure, changes in blood pressure, failure of circulation, convulsions, hyperexcitability to stimuli, hyperglycemia, and acidosis occur. Hemodynamic effects are dependent on drug, dose, route of administration, and species (Gleason, Gosselin, and Hodge, 1963; Grob, 1956; Heath, 1961; Holmstedt, 1959; and Koelle, 1965).

Measurement of Cholinesterase Activity

The reaction of acetylcholinesterase with its natural substrate, acetylcholine, forms the basis for the *in vitro* measurement of the enzyme's activity. Through the use of DFP, an irreversible inhibitor of the enzyme, the sequence and mechanism of action of the enzyme's active sites have been elucidated. The enzyme has 2 active sites: one, an anionic site bearing a unit charge and the second, an esteratic site containing a serine and a histidine residue. The quaternary N of acetylcholine forms an ionic bond with a dissociated carboxyl group at the anionic site while the carbonyl carbon of the substrate forms a covalent bond with the enzyme's serine residue at the esteratic site. The proton released in the reaction is accepted by the imidazoyl group of the histidine. Two hydrolysis reactions follow: the first releasing choline and acylated enzyme and the second releasing acetic acid and regenerated enzyme (Krupka, 1966; and Wilson, 1967). Equations for the reactions are as follows:

acetylcholine + enzyme ⟶ enzyme-substrate complex

enzyme-substrate complex + HOH ⟶ choline + acylated enzyme

acylated enzyme + HOH ⟶ acetic acid + enzyme

Measurement of enzymatic activity based on decrease in amount of added substrate can be made by reacting the remaining choline ester with alkaline hydroxylamine to form hydroxamic acid. The latter reacts with acid ferric chloride to form a brown-colored complex, whose absorbance is proportional to the amount of reacting substrate (Bonting and Featherstone, 1956; Hestrin, 1949; and de la Huerga, Yesinick, and Popper, 1952). By using thiocholine ester as substrate, Tabachnick (1956) measured decrease in substrate concentration of an enzyme-substrate mixture at 250 nm.

Measurements of enzymatic activity based on product formation can be divided into 2 categories: those measuring acetic acid or CO_2 released by acetic acid and those measuring thiocholine.

Manometrically, in a procedure devised by Ammon, the acetic acid produced by the action of enzyme on substrate can be reacted with $NaHCO_3$ in a Warburg flask and the amount of CO_2 released measured. Activity is expressed in µl of CO_2 evolved or µM of substrate hydrolyzed per 0.1 ml. of erythrocytes per 30 min. (Augustinsson, 1948).

Change in pH (ΔpH) of the reaction mixture caused by acetic acid production can be measured electrometrically with a glass electrode. The method, as published by Michel (1949), includes correction factors for Δ pH resulting from nonenzymatic hydrolysis of substrate and for change in enzyme reaction rate with decrease in pH. Enzyme activity is expressed as the rate of ΔpH/hr. Witter, Grubbs, and Farrior (1966) and Cestaric (1964) have introduced simplified modifications of this method.

By using indicators, ΔpH can be measured spectrophotometrically. Croxatto, Croxatto, and Huidabro (Augustinsson, 1957); Reinhold, Tourigney, and Yonan (1953); and Caraway (1956) used phenol red indicator. Rappaport, Fischl, and Pinto (1959) used m-nitrophenol. Limperos and Ranta (1953), using bromothymol blue, rated whole blood enzyme activity according to the color that developed in 20 minutes. Fleisher, Woodson, and Simet (1956), after modifying Limperos' and Ranta's test to measure the time required to reach a specific color, were able to detect a decrease of 50% or more of mean population activity in plasma (butyrylcholine substrate) or erythrocyte (acetyl-β-methyl choline substrate) enzyme activity.

Ellman *et al.* (1961) measured enzyme activity using acetylthiocholine as substrate. The thiocholine formed from substrate hydrolysis reacts with 5:5-dithiobis-2-nitrobenzoate (DTNB) to yield a yellow anion whose concentration is proportional to substrate consumed. By adding quinidine sulfate, an inhibitor of plasma esterase, to the reaction mixture, erythrocyte activity in whole blood was measured. Activity was expressed in moles of substrate hydrolyzed/min./RBC.

MATERIALS AND METHODS

The procedure of Ellman *et al.* (1961) was modified for use in the visual estimation of erythrocyte cholinesterase activity. The mean normal value of enzyme activity in moles of substrate hydrolyzed/min./ml. of blood and moles of substrate hydrolyzed/min./gm. of hemoglobin were calculated.

Blood from 38 male students was collected in heparin tubes.* Immediately after collection the blood samples were refrigerated (2-10 C.) and analyzed within 24 hours.

Hemoglobin concentration was measured on each sample by the cyanmethemoglobin method of Drabkin.

Erythrocyte cholinesterase activity was measured on each sample by the method of Ellman *et al.* (1961). The procedure was modified to include sterox** as a cell lysing agent in the phosphate buffer (pH 8.0, 0.1 M). (See Appendix I for reagent preparation.) Enzyme activity was calculated using the formulas:

moles of substrate hydrolyzed/min./ml. of blood =

$$(4.50)(10^{-5})\Delta A$$

moles of substrate hydrolyzed/min./gm. of hgb. =

$$(4.50)(10^{-3})\ \frac{\Delta A}{\text{Hgb.}}$$

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*B-D Vacutainers 3200 KA

**Sterox SE, Item #64049. Harleco. Hartman-Leddon Co., Philadelphia, Pa.

where $(4.50)(10^{-5})$ and $(4.50)(10^{-3})$ are conversion factors reflecting dilution, extinction coefficient of yellow anion, and changes in units;

ΔA = change in absorbance/min.;

Hgb. = concentration of hemoglobin in gm./100 ml. of blood.

Preparation of Test and Standard Reagent Strips

Strips, 6 x 54 mm., were cut from electrophoresis paper wicks.* Test reagent strips were prepared by adding DTNB (0.025 ml.), quinidine sulfate (0.01 ml.), and acetylthiocholine iodide (0.02 ml.) to strips at points 2, 16, and 31 mm. from one end. Standard reagent strips were prepared by adding glutathione (0.02 ml.) and DTNB (0.025 ml.) to strips at points 2 and 31 mm. from one end. Each strip was placed in a 10 x 75 mm. test tube, flash frozen in a mixture of dry ice and alcohol, and lyophilized for 3 hours. Following lyophilization, the test tubes containing the strips were sealed and stored at -5 C. until used.

Visual Quantitation of Enzyme Activity

Enzyme activity was estimated from time in minutes required for the enzymatic reaction to produce sufficient yellow anion to match a standard color. The extinction coefficient of the yellow-colored anion is 13,600 and therefore:

$$\text{moles/L. of standard/min.} = \frac{\Delta A}{13{,}600} \times 122 \times 1.25$$

where $122 = \frac{3.05}{0.025}$ = correction factor for dilution of DTNB;

$1.25 = \frac{0.025}{0.02}$ = correction factor for dilution of standard.

Standards representing colors produced by the average normal enzymatic reaction in 3 and 5 minutes were prepared.

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*Beckman #319329.

The average normal enzymatic reaction proceeded at the rate of 0.122 A./min. There are presently no available methods useful for stopping the reaction. Correction for color produced as a result of thiol material released from the cells required the addition of blood to both standard and test reagent mixtures. Therefore, the time required for the production of yellow anion sufficient to match a single standard was measured.

Repeated assays with visual matching of enzyme reaction colors to 3 and 5 minute standard colors and to a 3 minute standard color with one-half enzyme concentration were performed.

Reference Method

Whole blood, 0.005 ml., was added to 3.0 ml. of phosphate-sterox buffer in a 1 cm. cuvette. The photometer* slit was adjusted so that the absorbance (at 412 nm.) of the solution in the cuvette was zero. Quinidine sulfate (0.01 ml.), DTNB (0.025 ml.), and acetylthiocholine iodide (0.02 ml.) were added to the cuvette. Changes in absorbance (ΔA_{total}) were recorded for 5 minutes. The reaction of blood thiol groups with DTNB in a mixture of blood, buffer, and DTNB was similarly recorded (ΔA_{SH}). The time required for $\Delta A_{total} - \Delta A_{SH}$ to equal the absorbance of a standard was calculated.

Modified Visual Method

Whole blood, 0.005 ml., was added to each of 2 10 x 75 mm. test tubes. Each tube contained 3.0 ml. of phosphate-sterox buffer. At zero time a standard reagent strip was added to one tube; a test reagent strip was added to the other tube. Both tubes were vigorously shaken for 3

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*Beckman DU, Beckman Instruments, Inc., Fullerton, Calif. 92634.

minutes, the strips removed, and elasped time recorded when the color of the enzymatic reaction mixture matched that of the standard.

Temperature-Rate Studies

Five samples of blood were analyzed by the reference mthod at 15, 20, 25, 30, and 35 C. Factors relating rates to a 25 C. base temperature were calculated.

RESULTS

The mean normal value of erythrocyte cholinesterase activity in moles of substrate hydrolyzed/min./ml. of blood at 25 C. was $5.48 \times 10^{-6} \pm 0.57$ (1 s.d.). The mean normal value in moles of substrate hydrolyzed/min./gm. of hemoglobin at 25 C. was $3.49 \times 10^{-5} \pm 0.41$ (1 s.d.).

The stability of the strips impregnated with reagents was investigated following lyophilization and storage. The data in Table 1 indicate some deterioration in test reagent strips probably resulting from substrate instability. If adjustment is made for the initial decrease in absorbance and deterioration incurred during the lyophilization process, the absorbance contributed by reagent breakdown following 3.5 months of storage at -5 C. as compared to the total absorbance of the enzymatic reaction at 5 minutes is 7.83%. No significant deterioration of standard reagent strips occurred during 1 month of storage following lyophilization.

Results of repeated assays in which enzyme activity was estimated in minutes required to match a standard color are shown in Table 2. The data show that precision of visual matching, as indicated by the percentage $ts/\bar{x}$, was only slightly affected by increasing the intensity of the standard color. Increasing the time interval required to match the 3 minute standard color (enzyme concentration reduced to one-half) had only a slight effect on precision. In practice, it was found that at least 3 minutes were required to remove all of the standard from the reagent strip.

Seventeen samples of blood were assayed by the reference and modified methods. Results are shown in Table 3. The mean and standard deviation of the reference method was 5.19 $\pm$ 0.66 as compared to 5.08 $\pm$ 0.72 for the modified method. The index or error of discrimination (see Appendix II) at 55% of normal activity was 5.8%. The modified visual method had a precision of $\pm$ 25%.

Using the reference method, the effect of temperature on enzyme rate was investigated at 15, 20, 25, 30, and 35 C. Correction factors relating rates to 25 C. were calculated. The time of color change was related to temperature and level of cholinesterase activity at mean activity of the normal sample and 71% of mean activity (Figure 1).

TABLE 1. Comparison of reagent absorbances using fresh reagents and lyophilized strips

Sample	Number of determinations	Average initial absorbance	A./0.609*
		Test Reagents	
Fresh	2	0.0340	5.57%
Strips at 1 day	2	0.0438	7.20%
Strips at 1 month	2	0.0368	6.04%
Strips at 3.5 months	2	0.0917	15.05%
		Standard Reagents	
Fresh	11	0.659 ± 0.004**	100.00%
Strips at 1 week	10	0.658 ± 0.020	99.80%
Strips at 1 month	5	0.656 ± 0.012	99.50%

*5 min. A. of average normal enzymatic reaction

**s.d.

TABLE 2. Comparison of estimates of enzyme activity (in minutes) using 3 and 5 minute color standards and a 3 minute color standard with one-half enzyme concentration

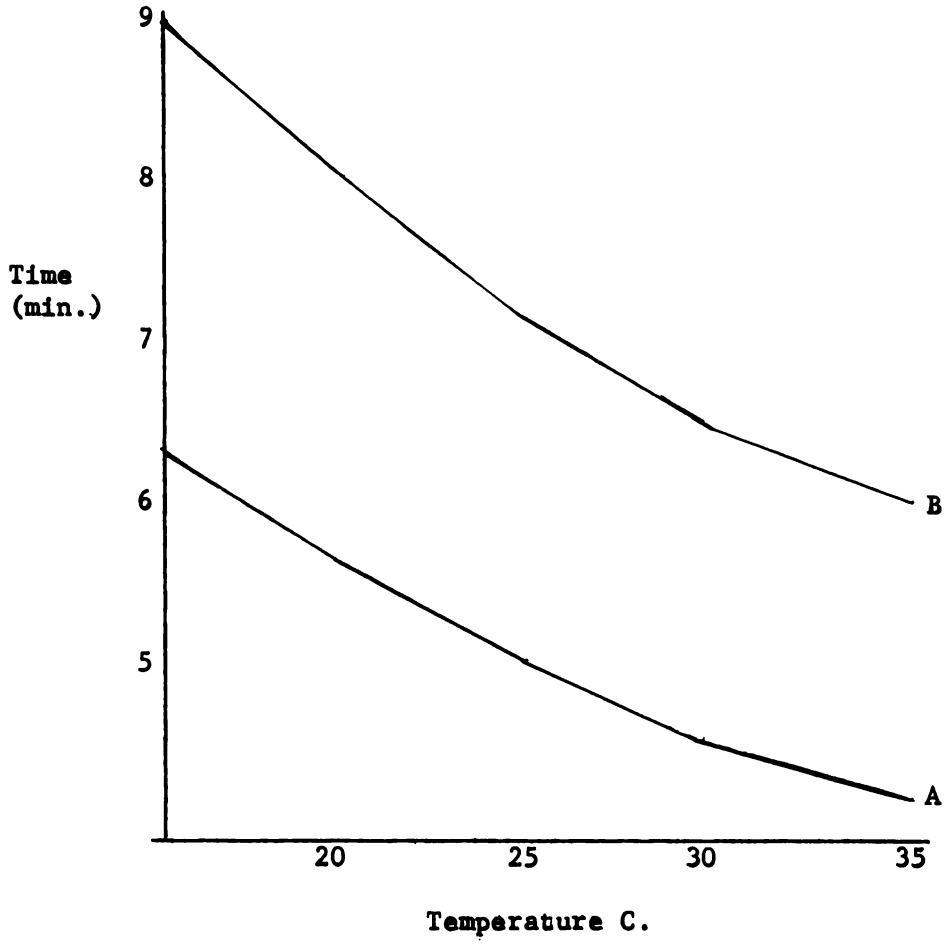
Standard	Mean estimate*	Standard Deviation	% precision
3 minute	3.06	0.30	22.2
5 minute	4.97	0.39	17.5
3 minute-1/2 enzyme	6.06	0.51	18.8

*N = 10

**$ts/\bar{x}$

TABLE 3. Comparison of reference method values with modified visual method values

Sample	Reference	Modified
1	4.38	4.60
2	5.64	5.02
3	5.53	5.50
4	6.10	5.17
5	4.40	5.18
6	5.14	5.27
7	5.33	5.22
8	4.56	3.43
9	5.63	5.50
10	6.25	5.87
11	4.28	5.67
12	5.57	6.15
13	5.51	4.83
14	4.65	4.97
15	4.92	5.05
16	6.01	5.47
17	4.38	3.47
$\bar{x}$	5.19	5.08
s	0.66	0.72
s^2	0.43	0.52



A = mean activity of normal sample
B = 71% mean activity

Figure 1. Time of color change in relation to temperature and level of erythrocyte cholinesterase activity at mean activity of normal sample and 71% mean activity.

DISCUSSION

Procedures that have been developed to date for the routine screening of cholinesterase activity either measure serum activity or use aqueous reagents lacking long term stability. Although toxic exposure to the organophosphate insecticides has a greater effect on plasma activity, regeneration of the plasma enzyme is a rapid process. The cumulative effects of repeated small exposures can be more reliably detected when erythrocyte activity is measured since the return of this enzyme activity to pre-exposure levels is a relatively slow process dependent on hematopoiesis. Decrease of 70% to 75% of original erythrocyte activity can be tolerated before symptoms of serious illness occur (Grob and Harvey, 1949). Therefore, a test permitting the measurement of erythrocyte activity was sought.

Lyophilized reagent strips provided premeasured reagents in a form convenient for use. Stability of aqueous substrate stored at refrigerator temperature does not exceed 15 days. Substrate deterioration on the strips at 3.5 months contributed only a small portion of the total absorbance of the enzymatic reaction. No corrections were made for substrate deterioration, nonenzymatic hydrolysis of substrate (0.0011 A./min.) or absorbance of materials other than sulfhydryl groups in the modified visual procedure. Adjustment of the standard would be required to correct for these reactions and should be considered in further work.

The original procedure published by Ellman *et al.* (1961) measured enzyme activity of whole erythrocytes. A mean value of 1.08×10^{-15} moles of substrate hydrolyzed/min./erythrocyte was found by this group. The addition of sterox to the phosphate buffer (pH 8.0, 0.1 M) as a cell lysing agent simplified visual and spectrophotometric reading of enzyme activity. Assuming a mean value of 29γγ of hemoglobin/erythrocyte and converting the moles of substrate hydrolyzed/min./erythrocyte from above to moles of substrate hydrolyzed/min./gm. of hemoglobin a value of 3.72×10^{-5} moles is obtained. This compares with a value of 3.49×10^{-5} moles obtained using the modified buffer. No effect was noted on linearity of reaction.

The sample of blood required for the test, 0.01 ml., can be obtained by ear or finger puncture. No specialized equipment is required. Enzyme activity can be estimated in less than 15 minutes with a precision of $\pm$ 25%.

There is a wide variation in erythrocyte cholinesterase activity from individual to individual. Levels within an individual, however, remain relatively constant, showing no variation in diurnal or seasonal studies (Callaway, Davies, and Rutland, 1951). Ideally individual base line values should be obtained prior to contact with anticholinesterase agents for comparison with later values. Practically the time calculated for 29% decrease in mean population normal activity can be used with a 5.8% index of discrimination. Observation of the test and standard tubes can be made at 7.16 minutes and activity considered as greater than, less than, or equal to 55% of normal value.

Erythrocyte cholinesterase concentration is dependent on total erythrocyte count. Decreased enzyme levels are usually seen only in pernicious anemia in relapse and paroxysmal nocturnal hemoglobinuria (Auditore and

Hartmann, 1959). Therefore, a decrease in activity cannot be attributed solely to exposure to anticholinesterase chemicals.

SUMMARY AND CONCLUSIONS

Repeated small exposures to the organophosphate insecticides, resulting in an increased susceptibility to poisoning, cause variable decreases in the activity of erythrocyte cholinesterase. A rapid, simplified procedure applicable for large scale survey testing in the detection of these small exposures was sought.

The procedure developed is a modification of the procedure of Ellman *et al.* and makes use of lyophilized reagent strips. Enzyme activity is visually estimated by measuring the time required for a reaction mixture of whole blood, buffer, and test reagent strip to match the color of a mixture of whole blood, buffer, and standard reagent strip.

Erythrocyte cholinesterase activity of 38 male college students in moles of substrate hydrolyzed/min./gm. of hemoglobin at 25 C. was $3.49 \times 10^{-5} \pm 0.41$ (1 s.d.) or in moles of substrate hydrolyzed/min./ml. of blood, $5.48 \times 10^{-6} \pm 0.57$ (1 s.d.).

Seventeen samples of blood assayed by the reference and modified visual methods had a mean and standard deviation of 5.19 ± 0.66 and 5.08 ± 0.72, respectively.

Lyophilized test reagent strips could be used for at least 3.5 months if stored at -5 C. Standard reagent strips showed no loss of activity after 1 month of storage under similar conditions.

The precision of visual estimation of enzyme activity was not affected by varying the intensity of the standard color or the rate of approach to a standard color.

Correction factors relating rate of enzyme reaction at temperatures ranging from 15-35 C. to a base temperature of 25 C. were calculated. The time of color change was related to temperature and level of erythrocyte cholinesterase activity at the mean activity of the normal sample and 71% mean activity.

A decrease in erythrocyte cholinesterase activity of 45% or more could be detected with a 5.8% index of discrimination. The method had a precision of $\pm$ 25%.

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APPENDICES

APPENDIX I

Reagents

1. Potassium dihydrogen phosphate, 0.1 M: Dissolve 13.609 gm. potassium dihydrogen phosphate in distilled water and dilute to 1 L.

2. Sodium hydroxide, 0.1 M: Dissolve 4.0 gm. sodium hydroxide in distilled water and dilute to 1 L.

3. Phosphate-sterox buffer, pH 8.0, 0.1 M: To 50 ml. of 0.1 M potassium dihydrogen phosphate add 46.1 ml. of 0.1 M sodium hydroxide. To each 100 ml. of buffer add 0.1 ml. of sterox.*

4. Phosphate buffer, pH 7.0, 0.1 M: To 50 ml. of 0.1 M potassium dihydrogen phosphate add 29.1 ml. of 0.1 M sodium hydroxide.

5. Acetylthiocholine iodide, 0.075 M: Dissolve 21.67 mg. acetylthiocholine iodide in 1 ml. distilled water.

6. Dithiobisnitrobenzoic acid (5:5-dithiobis-2-nitrobenzoic acid, DTNB), 0.01 M: Dissolve 39.6 mg. DTNB in 10 ml. pH 7.0, 0.1 M phosphate buffer. Add 15 mg. sodium bicarbonate.

7. Quinidine sulfate, 0.1%: Dissolve 100 mg. quinidine sulfate in distilled water and dilute to 100 ml.

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*Sterox SE, Item #64049. Harleco. Hartman-Leddon Co., Philadelphia, Pa.

8. Glutathione standard, 0.00683 M: Dissolve 20.97 mg. glutathione in distilled water and dilute to 20 ml. A mixture of 3.0 ml. phosphate-sterox buffer, 0.025 ml. DTNB, and 0.02 ml. glutathione standard should have an A. of 0.609 when read at 412 nm. in a 1 cm. cuvette against a water blank.

APPENDIX II

Data for Index of Discrimination

Index or error of discrimination = intersection of upper confidence limit of normal population and lower confidence limit of abnormal population (Lewis, 1966).

Normal population	Abnormal population
$\bar{x} = 5.08$	$\bar{y} = 9.24^*$
$s = 0.72$	$s = 0.72$
$n = 17$	$n = 17$

$$x = \bar{x} + \frac{ts}{\sqrt{n}} + Zs\sqrt{\frac{n-1}{X^2}}$$

$$y = \bar{y} - \frac{ts}{\sqrt{n}} - Zs\sqrt{\frac{n-1}{X^2}}$$

$Z = 1.57$

$p = .0582$

*By calculation 55% of average normal activity.

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