AD

EDGEWOOD ARSENAL
TECHNICAL REPORT

EATR 4607

AUTOMATED METHODS FOR MEASURING BLOOD CHOLINESTERASE: A SELECTIVE COMPARISON AND A NOVEL ARTIFACT

by

Robert I. Ellin, Ph.D.
William A. Groff
Andris Kaminskis

April 1972

DEPARTMENT OF THE ARMY
EDGEWOOD ARSENAL
Biomedical Laboratory
Edgewood Arsenal, Maryland 21010

16

Distribution Statement

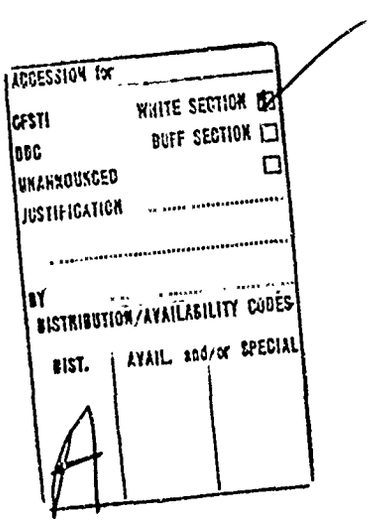
Approved for public release; distribution unlimited.

Disclaimer

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

Disposition

Destroy this report when no longer needed. Do not return it to the originator.



UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
CO, Edgewood Arsenal ATTN: SMUEA-BL-RCL Edgewood Arsenal, Maryland 21010	UNCLASSIFIED 2b. GROUP NA

3. REPORT TITLE

AUTOMATED METHODS FOR MEASURING BLOOD CHOLINESTERASE:
A SELECTIVE COMPARISON AND A NOVEL ARTIFACT

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

This work was started in February 1970 and completed in June 1971.

5. AUTHOR(S) (First name, middle initial, last name)

Robert I. Ellin
William A. Groff
Andris Kaminskis

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
March 1972	19	10

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO	EATR 4607
c. Task No. 1W062116AD1904	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
Techniques of Evaluating Effects of Chemicals, New Methods for Biological Assays	NA

13. ABSTRACT

Cholinesterase activity units obtained by the more commonly used Levine automated systems are not valid, as the standard calibration curve for the method is determined with an artificial standard--glutathione, either in the presence or absence of the substrate acetylthiocholine. In the presence of the substrate, glutathione reacts with the substrate via a transesterification mechanism. The slope of the calibration curve, and consequently activity units, would vary, depending on the rate of interaction. In the absence of substrate, as glutathione dialyzes about one-third as rapidly as thiocholine, activity units are obtained that are approximately three times greater than valid values. An acceptable automated procedure is recommended. Utility and sensitivity of selected automated cholinesterase systems are discussed.

14. KEYWORDS

Automated
Cholinesterases
Accuracy
Dialysis
Artifact
Artificial standard
International unit
Transesterification
Acetylglutathione

DD FORM 1 NOV 68 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

UNCLASSIFIED

Security Classification

EDGEWOOD ARSENAL TECHNICAL REPORT

EATR 4607

AUTOMATED METHODS FOR MEASURING BLOOD CHOLINESTERASE: A SELECTIVE COMPARISON AND A NOVEL ARTIFACT

by

Robert I. Ellin, Ph.D.
William A. Groff
Andris Kaminskis

Medical Research Division

April 1972

Task 1W062116AD1904

DEPARTMENT OF THE ARMY
EDGEWOOD ARSENAL
Biomedical Laboratory
Edgewood Arsenal, Maryland 21010

FOREWORD

The work described in this report was authorized under Task 1W062116AD1904, Techniques of Evaluating Effects of Chemicals, New Methods for Biological Assays. The experimental work was started in February 1970 and completed in June 1971. The experimental data are contained in notebooks MN-2147 and MN-2368.

DIGEST

Cholinesterase activity units obtained by the more commonly used Levine automated systems are not valid, as the standard calibration curve for the method is determined with an artificial standard--glutathione, either in the presence or absence of the substrate acetylthiocholine. In the presence of the substrate, glutathione reacts with the substrate via a transesterification mechanism. The slope of the calibration curve, and consequently activity units, would vary, depending on the rate of interaction. In the absence of substrate, as glutathione dialyzes about one-third as rapidly as thiocholine, activity units are obtained that are approximately three times greater than valid values.

An acceptable automated procedure is recommended. Utility and sensitivity of selected automated cholinesterase systems are discussed.

CONTENTS

AUTOMATED METHODS FOR MEASURING BLOOD CHOLINESTERASE: A SELECTIVE COMPARISON AND A NOVEL ARTIFACT

I. INTRODUCTION.

The possible restriction of DDT and other organochlorides probably will lead to the more extensive use of organophosphorus compounds as pesticides; consequently, an increased demand for testing acetylcholinesterase (AChE) activity in blood by clinical laboratories should be anticipated. Because of the large number of samples that can be expected and the problems arising from the manual analysis of red blood cell AChE, an automated analytical system that is accurate, rapid, and precise would be most desirable. Automated equipment can be run at the rate of 60 or more samples per hour, and precision in such systems is exceptional.

Numerous reports have described automated systems for the quantitative measurement of AChE in blood. An automated colorimetric system involving a *p*H change of a buffer with the indicator, phenol red, was first applied to the analysis of serum cholinesterase.[1] A more sensitive automated procedure for measuring blood AChE was introduced by Levine, Scheidt, and Nelson[2] and then by Humiston and Wright.[3] Both investigating teams employed Ellman[4] reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The latter, on reacting with thiocholine, the hydrolytic product of the substrate acetylthiocholine, releases the highly colored thionitrobenzoate anion. In another automated procedure, Groff, Mounter, and Sim[5] chose the Hestrin[6] method, which uses acetylcholine as substrate, to determine AChE activity.

One of the principal problems of the automated Ellman technique is the advocated use of an artificial standard in the nature of reduced glutathione. The true standard should be thiocholine iodide However, the purity of this compound, purchased from a reliable commercial source, was totally unacceptable.* Other investigators indicated they used either glutathione alone or glutathione in the presence of acetylthiocholine for preparing standard calibration curves.[7,8] Different calibration curves were obtained, depending on the conditions employed, enzyme activity values varied from one laboratory to another.

---

[1]Winter, G D Cholinesterase Activity Determination in an Automated Analysis System. Ann. N.Y. Acad. Sci. *87*, 629 (1960).

[2]Levine, J B., Scheidt, R A., and Nelson, J. A. An Automated Micro Determination of Serum Cholinesterase. Automation in Analytical Chemistry, Technicon Symposium. pp 582-585. Mediad, Inc., New York, New York. 1965

[3]Humiston, C G., and Wright, G J An Automated Method for the Determination of Cholinesterase Activity. Toxicol Appl. Pharmacol *10*, 467 (1967)

[4]Ellman, G L., Courtney, K. D., Andres, J., Jr., and Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity Biochem Pharmacol 7, 88 (1961)

[5]Groff W A., Mounter, L A and Sim, V. M A Multichannel Analytical System for Continuous Monitoring of Blood Cholinesterase Automation in Analytical Chemistry, Technicon Symposium Vol I, pp 498-502. Mediad, Inc., New York, New York 1966

[6]Hestrin, S The Reaction of Acetylcholine and Other Carboxylic Acid Derivatives With Hydroxylamine and Its Analytical Application J Biol Chem *180*, 249 (1949)

[7]Fowler P R., and McKenzie, J M. Detection of Mild Poisoning by Organophosphorus and Carbamate Pesticides Using an Automated Method for Cholinesterase Activity. Automation in Analytical Chemistry, Technicon Symposium. Vol I, pp 155-159 Mediad, Inc., New York, New York 1966.

[8]Ward, F P., and Hess, T L., Jr Automated Cholinesterase Measurements, Canine Erythrocytes and Plasma. Amer. J Vet Res. *32*, 499 (1971)

*The elemental analysis of carbon, hydrogen, iodine, and sulfur was unacceptable. The compound did not react with DTNB at *p*H values of 4.4 or 8.3

We find that the activity units obtained by the Levine-modeled automated systems are not valid. Reasons are presented, and proper methodology is recommended. Other parameters, such as utility and sensitivity of selected systems and the uniform reporting of activity units, are discussed.

II. PROCEDURES.

A. Automated Systems.

The Levine[2] method probably is used in a majority of laboratories. Sample, buffer, and acetylthiocholine substrate are mixed as a sample stream and dialyzed against the recipient stream containing Ellman reagent. In the Humiston[3] method, buffered Ellman reagent is added to the sample stream and mixed with enzyme and similar substrate prior to dialysis against a recipient buffer solution. Voss[9] used the Ellman reagent, but did not use a dialysis module. In the Groff[5] method, sample and substrate are mixed and incubated prior to dialysis. The unconsumed acetylcholine substrate dialyzes into a recipient stream, where it is mixed with alkaline hydroxylamine, acidified, and then reacted with ferric chloride.

B. Standard Calibration Curves for Glutathione and Thiocholine Iodide.

Solutions of glutathione (GSH) were prepared to give final concentrations ranging from $6 \times 10^{-4} M$ to $5 \times 10^{-3} M$ both in water and buffer at *p*H 7.0. To prepare a standard calibration curve for thiocholine, a stock concentration of acetylthiocholine was prepared in 0.2-*N* sodium hydroxide. Hydrolysis to thiocholine occurred instantly. The basic solution was neutralized with hydrochloric acid to *p*H 5 and brought to volume with water. Aliquots then were diluted with either water or buffer to obtain desired concentrations. Thiocholine iodide and GSH were assayed in the absence and presence of substrate by both the Humiston and Levine methods. Standard calibration curves in the Levine method are shown in figure 1. (Thiocholine was also prepared by treating acetylthiocholine with a 5% final concentration of hemolyzed red cells. Hydrolysis was complete within 20 minutes.)

C. Dialysis Manifold.

An automated system (figure 2) was constructed to measure the separate dialysis rates of GSH and thiocholine iodide (SChI). One can measure the sulfhydryl content of the sample stream before dialysis or the recipient stream after dialysis. Volumes, and consequently flow rates, of samples entering the sample side of the dialyzer were identical to the volumes and flow rates of material entering the recipient side. Similar volumes of color-forming reagent were added to dialyzed material exiting the recipient side of the dialyzer when dialysis occurred, or directly to the sample when there was no requirement for dialysis. Dialysis rates were compared from resulting absorbance values.

D. Thin-Layer Chromatography.

To 9.2 mg of reduced GSH and 29 mg of acetylthiocholine was added 1 ml of buffer, *p*H 8.3. Final concentrations were $1.5 \times 10^{-2} M$ and $5 \times 10^{-2} M$, respectively. After 2 minutes a 1-μl sample was placed on a silica gel thin-layer plate (purchased from Quantum Industries, Fairfield, New Jersey). An equivalent amount of reduced GSH, acetylthiocholine, and S-acetylglutathione were spotted separately on the same plate. The plate was developed for 2.5 hours with

[9]Voss, G. The Fundamental Kinetics of Cholinesterase Reaction With Substrate and Inhibition in an Automated Continuous Flow System. Residue Rev. *23*, 71 (1968).

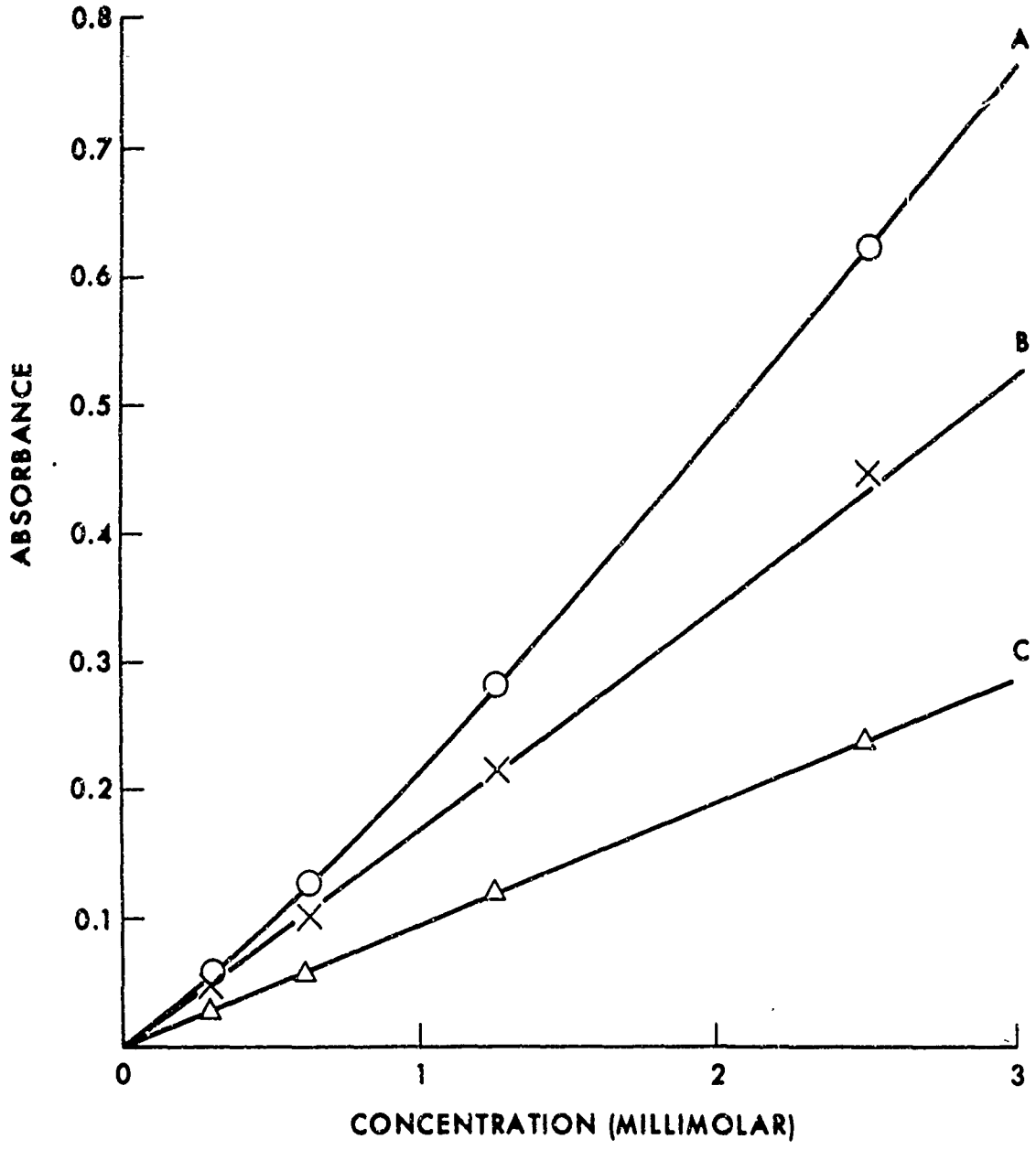


Figure 1. Calibration Curves for. (A) Thiocholine in *p*H 7 Tris Buffer, Assayed in the Presence of 20 mmoles Acetylthiocholine, (B) Glutathione in Water, Assayed in the Presence of 20 mmoles Acetylthiocholine; and (C) Glutathione in Water, Assayed in Absence of 20 mmoles Acetylthiocholine

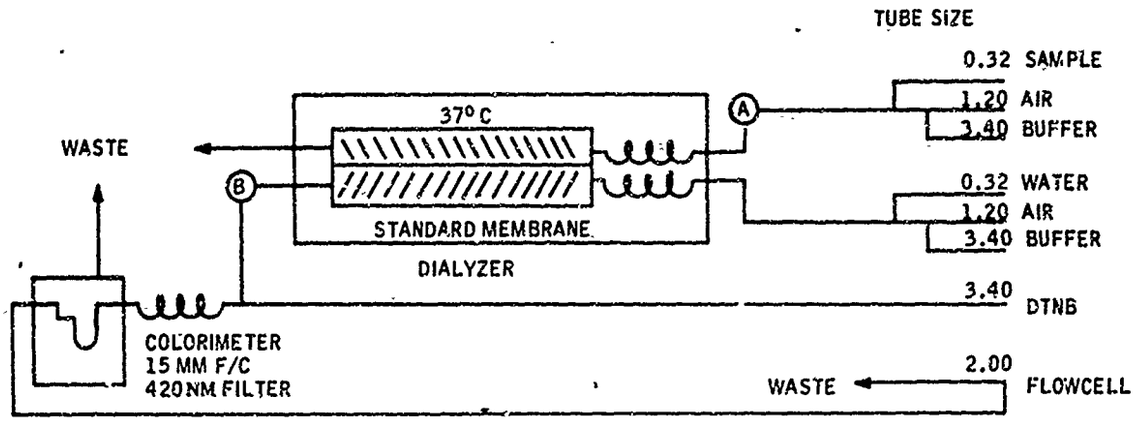


Figure 2. Special Manifold to Measure Percent Dialysis
As shown, measures dialysate. To measure before dialysis, connect A to B, by passing the dialyzer.

developing solvent, consisting of an equal mixture of butanol, pyridine, and water. Iodine vapors, 1% Ninhydrin reagent, and 0.5% Ellman reagent were used as spray visualization reagents.

E. Reaction of Glutathione With Acetylthiocholine Iodide

1. Glutathione With Excess Acetylthiocholine Iodide.

Stock solutions of 10^{-3} *M* GSH and 10^{-2} *M* acetylthiocholine iodide were prepared in distilled water. Initial concentrations to be assayed were prepared by diluting the stock solutions fivefold with Tris buffer, *p*H 8.2, and adjusting the final *p*H to 8.2, where necessary, using a 10% solution of sodium hydroxide. The buffered solutions were agitated for 15 minutes at 37°C before sampling and during the ensuing 5-minute sampling period. Assays were performed using the manifold shown in figure 2. Concentrations calculated from absorbancies of reactants and solutions shown in table I were investigated to determine whether or not a reaction had occurred.

2. Acetylthiocholine Iodide With Excess Glutathione.

Stock solutions of 10^{-3} *M* acetylthiocholine and 10^{-2} *M* GSH were prepared in distilled water. Conditions and procedures for the reactions were identical to those described previously.

F. Bimolecular Rate Constant of Transesterification.

An automated system was constructed to study the possible interaction between GSH and acetylthiocholine. Glutathione was mixed with Tris buffer at *p*H 8.2 and then with acetylthiocholine. After a 2.5-minute incubation at 37°C, the reaction mixture was dialyzed against a recipient stream of Ellman reagent in *p*H 8.2 Tris buffer. A rate constant for the reaction between GSH and acetylthiocholine was found by maintaining the GSH concentration constant at 1.8×10^{-4} *M* and varying the acetylthiocholine concentration as shown in table II. The reaction that followed, in a 15-mm flow cell at 420 nm, was a result of changes in absorbance related to the formation of thiocholine. The remaining GSH concentration was calculated and consequently substituted into the bimolecular rate equation: $k_2 = 2.3/At \log a/a - x$, where A is the acetylthiocholine concentration, t is time, and $a - x/a$ is the remaining GSH.

Table I. Absorbance Values for Reaction Between Glutathione and Acetylthiocholine (at 420 nm)

Solution	Concentration[a]	Absorbance
	M	
Acetylthiocholine iodide and glutathione	1×10^{-2} 1×10^{-3}	0.197
Acetylthiocholine iodide	1×10^{-2}	0.027
Acetylthiocholine iodide and 2% red cells[b]	1×10^{-3}	0.160
Red blood cells (2%)	–	0
Acetylthiocholine iodide plus glutathione	1×10^{-3} 1×10^{-2}	0.620
Glutathione	1×10^{-2}	0.540

[a]Initial concentrations.
[b]Preparation of thiocholine.

Table II. Conditions for the Reaction Between Acetylthiocholine and Glutathione

Glutathione concentration, 1.8×10^{-4} *M*; absorbance, 0.165; thiocholine 1.8×10^{-4}; absorbance, 0.480; temperature, 37°C; *p*H, 8.2; time, 2.6 min; and k_2 is the bimolecular rate constant, averaging 3.7×10^2 liters/mole/min.

Concentration acetylthiocholine	Absorbance	Thiocholine formed	Glutathione remaining	Rate
M		%		$k_2 \times 10^2$
3.2×10^{-5}	0.175	3.2	96.8	–
8×10^{-5}	0.185	6.4	93.6	3.3
1.6×10^{-5}	0.210	14.3	85.7	3.8
8×10^{-4}	0.335	44	56	3.8
2×10^{-3}	0.438	87	13	4.0
4×10^{-3}	0.480	100	0	–

III. RESULTS.

The slopes of the standard curves for GSH in the presence of acetylthiocholine iodide and thiocholine iodide are identical in the automated Humiston method, however, the values differ in the Levine method. If one assumes the standard calibration curve for thiocholine (figure 1) to be a straight line passing through the origin, although it is curved, then the slope for the thiocholine calibration is 2.5 times greater than the calibration slope for GSH. The difference in the slope values is directly proportional to differences in their respective rates of dialysis. About 10% of the total thiocholine present and 3.5% of GSH dialyze across the membrane. The slope of the standard calibration curve for GSH in the presence of acetylthiocholine is 1.9 times greater than that of GSH. Our results show conclusively that the latter phenomenon occurs because of an interaction between the two compounds to form thiocholine and acetylglutathione.

Table I. Absorbance Values for Reaction Between Glutathione and Acetylthiocholine (at 420 nm)

Solution	Concentration[a]	Absorbance
	M	
Acetylthiocholine iodide and glutathione	1×10^{-2} 1×10^{-3}	0.197
Acetylthiocholine iodide	1×10^{-2}	0.027
Acetylthiocholine iodide and 2% red cells[b]	1×10^{-3}	0.160
Red blood cells (2%)	–	0
Acetylthiocholine iodide plus glutathione	1×10^{-3} 1×10^{-2}	0.620
Glutathione	1×10^{-2}	0.540

[a]Initial concentrations.
[b]Preparation of thiocholine.

Table II. Conditions for the Reaction Between Acetylthiocholine and Glutathione

Glutathione concentration, 1.8×10^{-4} *M*; absorbance, 0.165; thiocholine 1.8×10^{-4}; absorbance, 0.480; temperature 37°C; *p*H, 8.2; time, 2.6 min; and k_2 is the bimolecular rate constant, averaging 3.7×10^2 liters/mole/min.

Concentration acetylthiocholine	Absorbance	Thiocholine formed	Glutathione remaining	Rate
M		%		$k_2 \times 10^2$
3.2×10^{-5}	0.175	3.2	96.8	–
8×10^{-5}	0.185	6.4	93.6	3.3
1.6×10^{-5}	0.210	14.3	85.7	3.8
8×10^{-4}	0.335	44	56	3.8
2×10^{-3}	0.438	87	13	4.0
4×10^{-3}	0.480	100	0	-

III. RESULTS.

The slopes of the standard curves for GSH in the presence of acetylthiocholine iodide and thiocholine iodide are identical in the automated Huggins method, however, the values differ in the Levine method. If one assumes the standard calibration curve for thiocholine (figure 1) to be a straight line passing through the origin, although it is curved, then the slope for the thiocholine calibration is 2.5 times greater than the calibration slope for GSH. The difference in the slope values is directly proportional to differences in their respective rates of dialysis. About 10% of the total thiocholine present and 3.5% of GSH dialyze across the membrane. The slope of the standard calibration curve for GSH in the presence of acetylthiocholine is 1.9 times greater than that of GSH. Our results show conclusively that the latter phenomenon occurs because of an interaction between the two compounds to form thiocholine and acetylglutathione.

Reactions between GSH and acetylthiocholine iodide were carried out first with an excess of acetylthiocholine, then with an excess of GSH. In experiments using excess acetylthiocholine, the absorbance values of the products resulting from the reaction were compared with that resulting from the enzymatic hydrolysis of an equivalent concentration of acetylthiocholine. Table I shows the following results. (1) absorbance from thiocholine iodide produced by the transesterification reaction and spontaneous hydrolysis of acetylthiocholine, (2) absorbance from the spontaneous hydrolysis of acetylthiocholine iodide at *p*H 8.2; (3) absorbance of thiocholine produced by enzymatic hydrolysis, (4) absorbance of 2 vol % red blood cells, (5) absorbance resulting from both thiocholine iodide formed by transesterification and the excess GSH, and (6) absorbance as measure of 10^{-2} *M* GSH.

Using the absorbance values shown in table I, observed results agreed to within 6% of those calculated when excess acetylthiocholine was reacted with GSH and to within 4% when excess GSH was reacted with acetylthiocholine. After completion of the latter reaction, red blood cells were added. No additional thiocholine, which would form by the enzymatic hydrolysis of acetylthiocholine, was produced.

The course of the reaction between GSH and acetylthiocholine was followed by thin-layer chromatography. Of eight developing solvent systems tested, an equal mixture of butanol, pyridine, and water gave the best separation of the products from the ensuing reaction. Our preliminary experiments showed that the developing solvent could separate GSH from S-acetylglutathione, however, the small separation of 0.05 RF units was not considered sufficient for positive identification. The Ellman reagent, however, can distinguish between the two as it produces a yellow color with GSH and no color with S-acetylglutathione. Additional spray reagents, such as 1% Ninhydrin (which gives a violet color with both glutathione and S-acetylglutathione) and iodine vapors that give color with all initial reaction compounds as well as reaction products were also used for identification. Our results showed the disappearance of GSH with the concomitant appearance of S-acetylthiocholine. The biomolecular rate constant for the reaction between acetylthiocholine and GSH at *p*H 8.2 and 37°C was calculated to be 3.7×10^2 liter/mole/minute (table II).

IV. DISCUSSION AND CONCLUSIONS.

In order to construct a versatile and universally acceptable automated system for measuring blood AChE activity, one that could be used for red cell and plasma enzyme and for blood samples that are drawn from a vein or fingertip, the most commonly used automated systems and procedures have been studied and evaluated. Most automated systems use a dialyzer assembly with a cellophane membrane to prevent protein from interfering with the spectrophotometric absorbance of the substance being measured. The importance of the dialysis step has been grossly underestimated and should be considered as thoroughly as any other part of the system. Most of the methods investigated include the dialyzer module, the Voss method does not. Concentration and chemical composition of materials on the sample side of the dialyzing plate and selected flow rates have decided effects on amounts dialyzing across the cellophane membrane.[10] Had calibration curves of both GSH and acetylthiocholine been more thoroughly investigated, interaction between the two to form thiocholine and S-acetylglutathione could probably have been resolved years ago.

The method described by Levine, Scheidt, and Nelson[2] is most commonly used. It has good precision and good sensitivity, and it provides low blank values. The weakness of this procedure lies in the preparation of a standard calibration curve. The substitution of GSH for the true standard, thiocholine, creates a problem. If an investigator chooses to obtain a standard curve

[10]Groff, W. A., and Ellin, R. I. A New and Rapid Determination of Pyridinium Oximes in Blood and Urine. Clin. Chem. *15*, 22 (1969).

using GSH in the absence of substrate,[8] the slope of the resulting calibration curve is approximately one-third that of the calibration curve of thiocholine. If GSH is measured in the presence of substrate, the calibration curve could measure the sulfhydryl product remaining, as well as those products by the transesterification reaction. Consequently, the slope of standard calibration curves would depend upon conditions that control the rate of interaction. Resulting data and acceptable normal and average cholinesterase values would vary from laboratory to laboratory. A similar observation was reported by Fowler and McKenzie.[7] These authors ruled out chemical interaction because manually performed experiments did not support this assumption. In the manual transesterification reaction, thiol equivalents do not change, regardless of how complete or incomplete the interreaction. Thus, a manual method would not show changes in absorbance. This problem can be resolved by using thiocholine, enzymatically or chemically converted from acetylthiocholine for a standard in a calibration curve. Thiocholine purchased from a commercial laboratory was not thiocholine. Fowler and McKenzie[7] suggest the use of chemical hydrolysis of acetylthiocholine and report stability at -20°C for months.

The other problem, which cannot be remedied as easily, is the possible interreaction of either dialyzable or nondialyzable sulfhydryl material present in biological samples. A reaction of the latter with acetylthiocholine would result in higher values in a red cell AChE assay. A similar error could result with dialyzable sulfhydryl because the proper correction factor depends on its rate of interaction with substrate as compared to its rate of dialysis and the interaction with DTNB. Neither of these potential sources of error can be corrected for in this procedure.

As Voss[9] did not use a dialyzer, the color of the DTNB and also red cells caused the blank readings to be high. Even in a 1:1200 aqueous dilution of red cells, the absorbance, because of enzyme activity after 16 minutes, was less than twice that of the blank absorbance. The inherent high blanks rule out the use of the procedure for red cell enzyme activity. This method can be used to monitor the enzyme in serum or tissues, which after homogenization is relatively low in sulfhydryl content.

Blank values caused by red cell interference are nil in the Groff[5] method—so much so that duplicate determinations to measure interference from the red cells do not have to be run. The method using the Hestrin[6] procedure is much less sensitive than the Ellman method because of the lower substrate specificity and lower molar extinction of color-producing reagent. A disadvantage of the Hestrin system that may be more disconcerting in an automated system than in a manual system is that it is a difference method and involves hydrolysis of a significant portion of substrate. Another problem that has not been resolved is that the method does not show changes in activity of red cell AChE after approximately 80% to 85% of the enzyme has been inhibited.

In the Humiston[3] method, the color-forming reagent is on the sample side of the dialyzer. The slopes of the standard calibration curves for GSH or thiocholine, even in the presence of substrate, are identical. Both dialyzable and nondialyzable sulfhydryl compounds react prior to dialysis. One could obtain reliable blank values in whole blood and red cell AChE determination. Blank values in this method are not nearly as large as that of the Voss method in which a dialyzer assembly is not used. The relatively high blank in the Humiston method can be subtracted from sample values. Consequently, we feel that this system is the method of choice.

Cholinesterase values may differ for each laboratory, not only because of the various ways enzyme activity is reported, but also because of the use of different experimental conditions. Temperature, *p*H, the nature of substrate, and substrate concentration should be uniform in order to obtain comparable data, even if all laboratories adopt the international unit for reporting their data. The international unit would be expressed as: one unit of enzyme catalyzes the transformation of 1 μmole of substrate per milliliter of sample per minute. Time is determined by measuring the total time that the sample and substrate react, measuring the time it enters the incubator bath and when it leaves the dialyzer plate. We suggest that conditions as near to *p*H 8.2,

Reactions between GSH and acetylthiocholine iodide were carried out first with an excess of acetylthiocholine, then with an excess of GSH. In experiments using excess acetylthiocholine, the absorbance values of the products resulting from the reaction were compared with that resulting from the enzymatic hydrolysis of an equivalent concentration of acetylthiocholine. Table I shows the following results. (1) absorbance from thiocholine iodide produced by the transesterification reaction and spontaneous hydrolysis of acetylthiocholine, (2) absorbance from the spontaneous hydrolysis of acetylthiocholine iodide at *p*H 8.2, (3) absorbance of thiocholine produced by enzymatic hydrolysis, (4) absorbance of 2 vol % red blood cells, (5) absorbance resulting from both thiocholine iodide formed by transesterification and the excess GSH, and (6) absorbance as measure of 10^{-2} *M* GSH.

Using the absorbance values shown in table I, observed results agreed to within 6% of those calculated when excess acetylthiocholine was reacted with GSH and to within 4% when excess GSH was reacted with acetylthiocholine. After completion of the latter reaction, red blood cells were added. No additional thiocholine, which would form by the enzymatic hydrolysis of acetylthiocholine, was produced.

The course of the reaction between GSH and acetylthiocholine was followed by thin-layer chromatography. Of eight developing solvent systems tested, an equal mixture of butanol, pyridine, and water gave the best separation of the products from the ensuing reaction. Our preliminary experiments showed that the developing solvent could separate GSH from S-acetylglutathione, however, the small separation of 0.05 RF units was not considered sufficient for positive identification. The Ellman reagent, however, can distinguish between the two as it produces a yellow color with GSH and no color with S-acetylglutathione. Additional spray reagents, such as 1% Ninhydrin (which gives a violet color with both glutathione and S-acetylglutathione) and iodine vapors that give color with all initial reaction compounds as well as reaction products were also used for identification. Our results showed the disappearance of GSH with the concomitant appearance of S-acetylthiocholine. The biomolecular rate constant for the reaction between acetylthiocholine and GSH at *p*H 8.2 and 37°C was calculated to be 3.7×10^2 liter/mole/minute (table II).

IV. DISCUSSION AND CONCLUSIONS.

In order to construct a versatile and universally acceptable automated system for measuring blood AChE activity, one that could be used for red cell and plasma enzyme and for blood samples that are drawn from a vein or fingertip, the most commonly used automated systems and procedures have been studied and evaluated. Most automated systems use a dialyzer assembly with a cellophane membrane to prevent protein from interfering with the spectrophotometric absorbance of the substance being measured. The importance of the dialysis step has been grossly underestimated and should be considered as thoroughly as any other part of the system. Most of the methods investigated include the dialyzer module, the Voss method does not. Concentration and chemical composition of materials on the sample side of the dialyzing plate and selected flow rates have decided effects on amounts dialyzing across the cellophane membrane.[10] Had calibration curves of both GSH and acetylthiocholine been more thoroughly investigated, interaction between the two to form thiocholine and S-acetylglutathione could probably have been resolved years ago.

The method described by Levine, Scheidt, and Nelson[2] is most commonly used. It has good precision and good sensitivity, and it provides low blank values. The weakness of this procedure lies in the preparation of a standard calibration curve. The substitution of GSH for the true standard, thiocholine, creates a problem. If an investigator chooses to obtain a standard curve

[10]Groff, W. A., and Ellin, R. I. A New and Rapid Determination of Pyridinium Oximes in Blood and Urine. Clin. Chem. *15*, 22 (1969).

37°C, and 1 mM acetylthiocholine substrate be used for red cell enzyme, and that enzyme activities be expressed as micromoles of substrate hydrolyzed per minute per milliliter of sample. We will then have the semblance of a method that will provide enzyme activity data that can be compared with those of other laboratories.

On the basis of this report, a modified automated system has been developed in our laboratory. It is precise and accurate; can be used for whole blood, red cell, and plasma enzyme with autodilution; and can handle samples as small as 20 μliters when diluted manually. A detailed report on this procedure has been submitted for publication