A COMPARISON OF THREE SERUM CHOLINESTERASE METHODS

Charles R. Crane, Ph.D.
Donald C. Sanders, M.S.
John K. Abbott, B.A.

Approved by

J. Robert Dille, M.D.
Chief, Civil Aeromedical Institute

Released by

P. V. Siegel, M.D.
Federal Air Surgeon

August 1970

Department of Transportation
FEDERAL AVIATION ADMINISTRATION
Office of Aviation Medicine

Qualified requesters may obtain Aviation Medical Reports from Defense Documentation Center. The general public may purchase from Clearinghouse for Federal Scientific and Technical Information, U.S. Dept. of Commerce, Springfield, Va. 22151.

A COMPARISON OF THREE SERUM CHOLINESTERASE METHODS

I. Introduction.

In recent years, clinical laboratories have received an increasing number of requests to perform blood cholinesterase (CHE) assays. This is primarily due to recognition of the relationship between the activity of this enzyme and an individual's exposure to organophosphate pesticides. A depression of CHE activity indicates absorption of the pesticide, and in many cases this depression will appear before symptoms or physical impairment occur. This assay therefore serves as both a diagnostic tool and as an "early warning device" for monitoring the health-safety aspects of a pesticide operation. Recognizing the value of such monitoring, the Health Department of at least one state now requires periodic blood CHE assays for all aerial applicator personnel during the spraying season.

Numerous methods have been used for the measurement of CHE activity, ranging from very precise assays requiring the use of Warburg or constant-pH titration (pH-Stat) equipment to simple field methods utilizing paper test strips. The units of activity obtained by these different methods are varied and are not readily interconvertible. Examples of these units include change in pH per unit time, change of a pH indicator with time, the time required for color development of a test strip to match the color of a control, as well as the more conventional unit: micromoles of substrate hydrolyzed/min/ml serum, as provided by the pH-Stat method. Other methods utilize units derived from color-coupling reactions with the products from unnatural substrates, and hydroxylamine reaction with the substrate (ester) remaining after incubation.

The clinical interpretation of a single CHE value as a measure of exposure can be difficult in some cases. The difficulty arises from the fact that the range of serum CHE values from a "normal" population of unexposed individuals extends above and below the population mean by as much as 30%. For this reason, the decrement in enzyme activity due to organophosphate exposure will be more meaningful if an individual's normal (pre-season) value is known. This is the basis for the recommendation that all applicator personnel have a pre-season cholinesterase test.

The comparison of a pre-season value with one determined at some later date is more easily interpreted if both values were determined by the same analytical method, but the peripatetic nature of aerial applicators and the diversity of cholinesterase methods used by clinical laboratories makes this unlikely. Consequently, a technique for converting from the activity units obtained by one analytical method to those of another is required.

This paper represents the first in a series which will provide conversion data to transform cholinesterase activity units obtained by one method into the corresponding units of another.

The general method used to develop these comparison data was to perform simultaneous assays by each method on aliquots of the same sample of serum, using the pH-Stat method as a primary reference.[1-3]

II. pH-Stat Reference Method.

Materials and Method. Butyrylcholine iodide (Mann Research Laboratories, New York, N.Y.) was dried overnight at 110°C and stored in a desiccator over Drierite. An aqueous solution, 0.163M (49 mg/ml), was prepared weekly and stored at 4°C. The sodium hydroxide titrant was prepared in a concentration of approximately 0.005N using CO_2-free distilled water. The solution was stored in a titrant reservoir of the pH-Stat and protected from CO_2 absorption by a drying tube filled with Mallcosorb, 30-50 mesh, indicating-type CO_2 absorbent (Mallinckrodt). This solution was standardized daily by titration with potassium hydrogen phthalate, primary standard grade. Ringer's salts solution for diluting serum samples was prepared as needed

using 0.9 g NaCl, 0.02 g KCl, 0.026 g $CaCl_2$, and 0.02 g $NaHCO_3$, brought to 100 ml final volume using distilled water.

A Radiometer pH-Stat (Radiometer A/S; Copenhagen, N.V., Denmark) was used for all constant-pH titrations, and consisted of the following units:

PHM26—expanded scale pH meter

TTT11b—electronic titration control unit

ABU1b—semi-micro automatic buret for titrant delivery, 2.5 ml capacity

TTA3—glass-jacketed reaction vessel, 3 ml, with plastic-coated magnetic stirring disc

VTS13c—constant-temperature water bath and circulator, set to maintain 25°C in reaction vessel

SBR2c—recorder for indicating volume of titrant delivered per unit time

Serum from freshly-drawn whole blood was purchased from a local blood bank. Part of the serum was heated in a water bath at 60°C until pH-Stat assay indicated a loss of about 95% activity (10–20 minutes). The inactivated serum was then mixed in varying proportions with fresh serum to obtain a gradient of enzyme activity. Aliquots of these samples were diluted 1:10 with Ringer's salts solution.

One ml of the diluted serum was added to the reaction vessel, the magnetic stirrer was started and the solution was allowed to come to temperature equilibrium (25°C). The pH was adjusted with the automatic titrator to the pre-set value of 8.1. One-half ml of butyrylcholine iodide substrate was added to the reaction vessel using a 1.0 ml tuberculin syringe and the recorder chart drive was turned on. The reaction was allowed to continue until the recorder had plotted a straight line (titrant volume *vs* time) for a minimum of 3 minutes, or until a minimum titrant volume of 0.5 ml had been added.*

* Since full scale on the recorder (100 scale divisions) represents 2.5 ml of titrant, 0.5 ml represents 20 scale divisions. The recorder trace can be read to 0.2 scale division; therefore, the precision in reading titrant volume is 1%. With a chart speed of 2 cm per min, the precision in reading elapsed time of 3-min trace is also 1%.

Calculations:

All activities were expressed as micromoles of substrate hydrolyzed/minute/ml of serum. Calculations were made as follows:

$$\text{Act.} = \frac{\text{S.D.}}{t} \times (0.025) \times (N) \times (1000\ \mu\text{moles/mmole}) \times (\text{D.F.})$$

Where S.D. = recorder scale divisions (linear portion)

t = time in minutes for linear portion of trace

0.025 = ml titrant per S.D.

N = normality of titrant

D.F. = dilution factor (i.e., 10)

III. Boehringer Method.

The Boehringer method for the determination of serum CHE activity is a colorimetric method utilizing acetylthiocholine iodide as a substrate. The thiocholine released by the enzyme couples with 5,5′-dithiobis-2-nitrobenzoate (DTNB, dithiobisnitrobenzoate) to form a yellow product with an absorbance maximum at 405 nm. Historically, the method was developed by Ellman et al.[4] by combining his earlier work using DTNB for the detection of tissue sulfhydryl groups[5] and the work of Koelle[6] who used acetylthiocholine as a substrate for a histochemical CHE determination. Garry and Routh[7] modified the original method by stopping the reaction with excess quinidine sulfate after three minutes of incubation. Levine[8] and Humiston[9] utilized the reaction in the development of automated methods.

Materials and Method. All materials used for the assay were supplied in the Boehringer Kit TC-CE, Catalog No. 15984TCAB (C. F. Boehringer & Soehne, GMBH, Mannheim, Germany**) and the method outline supplied with the kit was followed. Spectrophotometric measurements were performed using a Beckman DU monochromator attached to a Gilford Model 222 photometer and power supply. Absorbances were recorded with a Varian Model G–14A–3G strip chart recorder operated at 1 inch/min with a full scale operating range of 0–1 absorbance.

Individual assays were performed by pipetting directly into a 1 cm cuvette 3.0 ml of 0.0002M

** Available from Boehringer Mannheim Corp., 219 East 44th Street, New York, N.Y. 10017.

DTNB in 0.05M aqueous phosphate buffer (pH 7.2) and 0.02 ml of the serum sample. The substrate, 0.10 ml of 0.156M acetylthiocholine iodide, was added and rapidly mixed with a plastic paddle; the cuvette was introduced into the light path and the recorder started. The increase in absorbance at 405 nm was recorded for a 3-minute period after the trace became linear. The rate of absorbance increase was expressed as $\triangle$ A/30 sec, and this rate was multiplied by the factor 23,400 to obtain the units of enzyme activity as Boehringer expresses them, namely milliunits/ml serum.

Since we were interested in deriving conversion factors for use in the average clinical laboratory, a themostated cuvette chamber was not used. However, the substrate and buffer solutions were kept in a 25°C water bath until just before use and the temperature variation during the 3-min recording was less than 0.5°C. Correction for non-enzymatic hydrolysis was based on a blank reaction using water instead of serum, but was usually zero for a 3-min period.

Calculated Boehringer milliunits were plotted against pH-Stat units obtained for the same serum samples assayed simultaneously. The linear regression equations, standard errors of the estimates and correlation coefficients were calculated, each point representing the mean of duplicate assays of the same sample by each method. The statistical regression of the pH-Stat units on Boehringer milliunits is graphically represented in Figure 1.

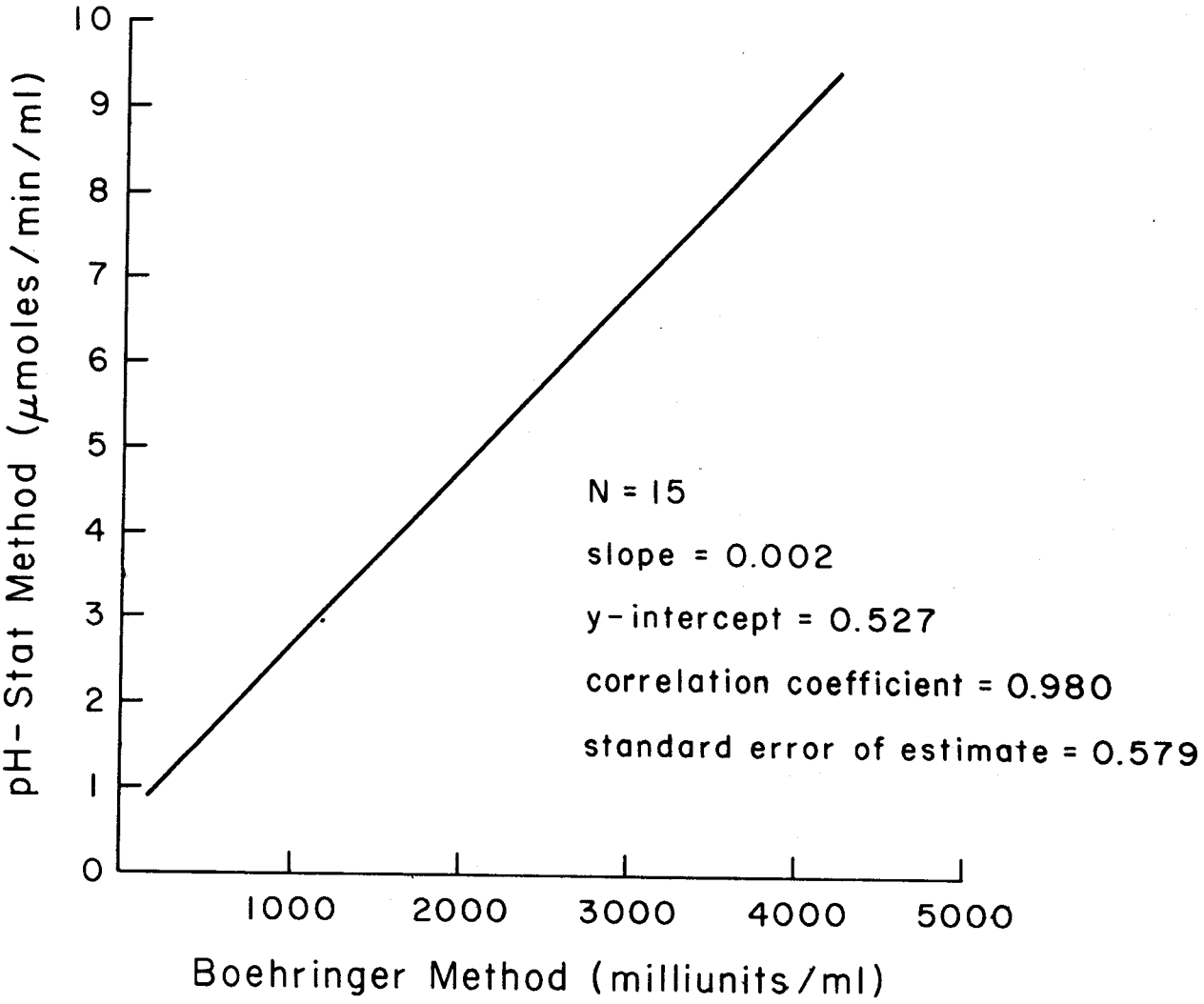

FIGURE 1. Nomogram for the interconversion of cholinesterase activity values: pH-Stat units—Boehringer milliunits.

IV. Sigma Method.

The Sigma method utilizes the acid-base indicator *m*-chlorophenol to measure colorimetrically the decrease in pH of the reaction mixture resulting from the liberation of acetic acid from acetylcholine. The procedure is essentially that of Rappaport, Fischl, and Pinto[10] and differs from similar early procedures principally in the substitution of *m*-chlorophenol for phenol red as an indicator[11 12]. This substitution avoids the "protein error" which would be caused by the selective binding of phenol red to serum proteins, and the consequent shift in wave-length of the absorption maximum.

Materials and Method. Materials used in this assay were supplied in Sigma Kit 420–CC (Sigma Chemical Company, 3500 DeKalb Street, St. Louis, Missouri); the assays were performed as described in Sigma Tentative Technical Bulletin No. 420 (revised July 1967). Colorimetric measurements were performed on a Gilford Model 300 spectrophotometer.

An individual assay tube contained the following components:

- 0.2 ml serum
- 0.2 ml saline (0.9%)
- 3.0 ml distilled water
- 2.0 ml *m*-nitrophenol solution (in phosphate buffer at pH 7.8, Sigma Stock Solution #420–2, Reagent B)
- 0.2 ml acetylcholine chloride solution (150 mg/ml)

A blank tube was prepared for each assay by heating the serum-saline mixture for 5 minutes in a 60°C water bath, then adding the remaining components. Blank and assay tubes were incubated at 25°C for exactly 30 minutes following the addition of substrate. The absorbances were measured at 420 nm and the absorbance of the assay tube was subtracted from that of the blank, using distilled water as a reference. The resulting values were compared to a calibration curve prepared by substituting 4, 8, 12, 16, and 20 μmoles of acetic acid for the 0.2 ml of substrate; these quantities correspond to 20, 40, 60, 80 and 100 Rappaport units respectively.

Rappaport units were plotted against pH-Stat data for corresponding serum samples and the regression equations, correlation coefficients, and standard errors of the estimates were calculated. The statistical data for the regression of the pH-Stat units on Rappaport units are shown in Figure 2.

We did not find the 5-minute denaturation at 60°C, which is recommended by Sigma, to be adequate to inactivate the enzyme completely in the blank solution. Serum samples diluted 1:1 with isotonic saline and heated to 60°C retained approximately 6% of their original activity at the end of 5 minutes, and approximately 2% after 10 minutes of heating when assayed by the pH-Stat method.

Furthermore, although Sigma advises that heparinized plasma may be substituted for serum in this assay, we found that the heat denaturation step caused turbidity in the plasma sample and precluded accurate colorimetric measurement unless the sample was clarified by filtration or centrifugation. Since the △ absorbance used to determine the enzyme activity from the standard curve is obtained by subtracting the absorbance of the assay tube, which is not denatured, from that of the blank tube, which has been denatured, it is obvious that turbidity in the blank will materially increase the number of Rappaport units read from the standard curve.

To determine the extent of this turbidity error on serum *vs* plasma measurements, blood samples from the same individual were drawn into heparinized and non-heparinized tubes and the corresponding plasma and serum fractions were prepared. These two preparations were then assayed by the Sigma method and by the pH-Stat method. Since the latter is a titrimetric method, the turbidity in the plasma preparations is of no consequence. pH-Stat values for the two preparations agreed within 1% of each other, but the corresponding colorimetric Rappaport units were 103 for serum and 210 for plasma.

In an additional experiment, 1.0 ml aliquots of a Sigma assay mixture prepared from plasma were transferred to a pH-Stat reaction vessel immediately after addition of substrate. These were allowed to react for 30 minutes at 25°C. At the end of the reaction period, the aliquot was quickly back-titrated with standardized base to its original pH, and the equivalent Rappaport units (μmoles/ml/30 min) calculated. Values for duplicate determinations were 71.1 and 72.5 R.U. when determined by this titration technique using

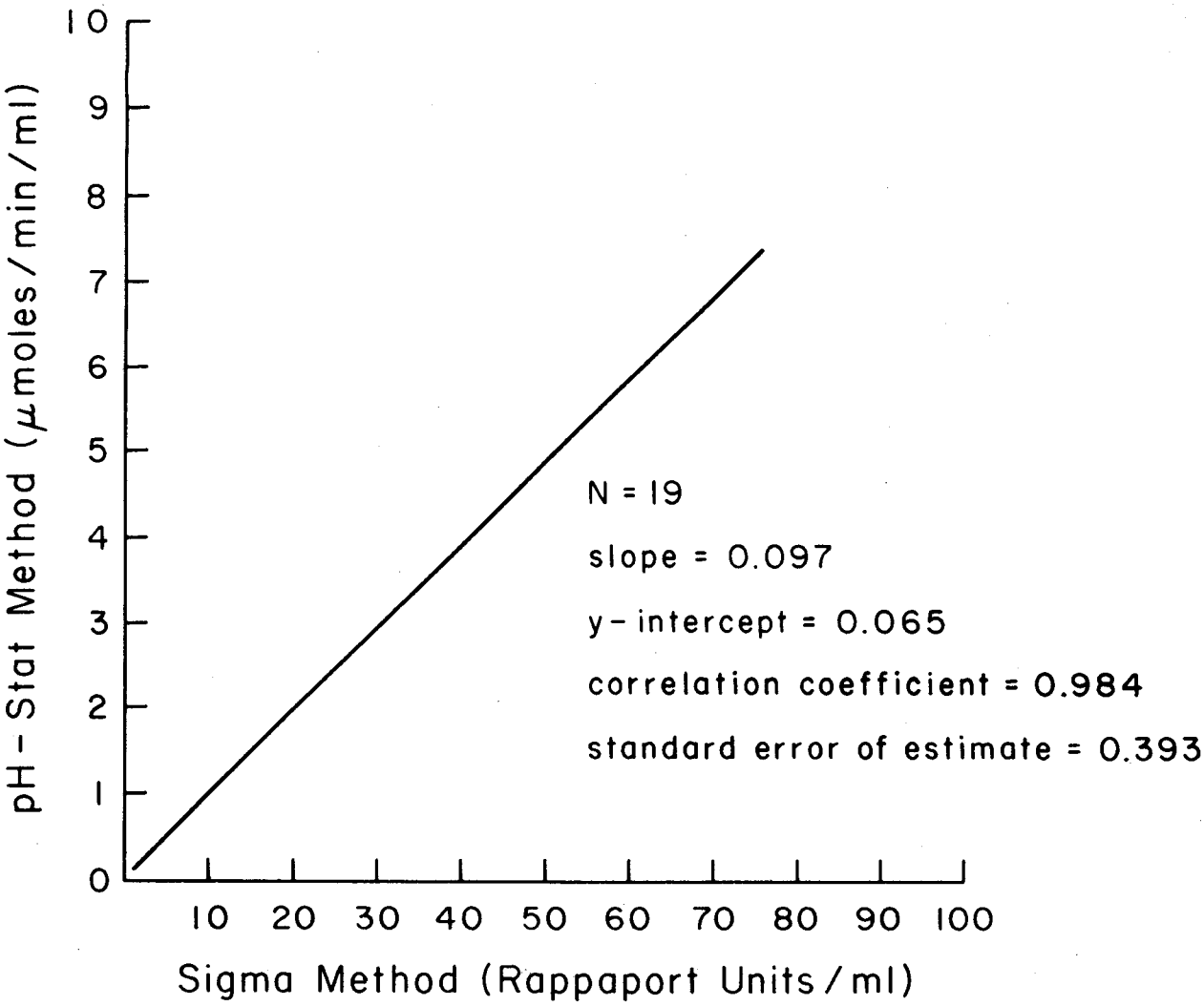

FIGURE 2. Nomogram for the interconversion of cholinesterase activity values: pH-Stat units—Rappaport units.

Sigma reagents. When additional aliquots of this same plasma mixture were assayed colorimetrically, following Sigma instructions, the corresponding values were in excess of 200 R.U. Thus, the difference between 71 and 200 R.U. is the error produced by the turbidity in the heat-denatured blanks used in the colorimetric method.

V. Conclusions.

The range of values from replicate determinations by any of the three methods was considerably less than the normal daily variation in plasma CHE activity for an individual.

We cannot recommend, however, the use of the Sigma kit method for determining plasma cholinesterase unless the heat-denatured plasma used in both the blank and the calibration curves is centrifuged to remove turbidity prior to colorimetric measurement.

Regression equations were calculated from the experimental data for the following conversions:

a. To convert Boehringer milliunits (BMU) to pH-Stat Units (see Figure 1):

pH-Stat Units = (0.002) BMU + 0.527 (1)

b. To convert Rappaport Units (Sigma Method) to pH-Stat Units (see Figure 2):

pH-Stat Units = (0.097) Rappaport Units + 0.065 (2)

c. To convert pH-Stat Units to Boehringer milliunits:

Boehringer milliunits = (458) pH-Stat Units − 156 (3)

d. To convert pH-Stat Units to Rappaport Units (Sigma Method):

Rappaport Units = (9.967) pH-Stat Units + 0.780 (4)

Conversion equations for the relationship between Rappaport Units and Boehringer milliunits were derived from equations (1) and (2). Thus:

e. To convert Boehringer milliunits to Rappaport Units:

Rappaport Units = (0.022) BMU + 4.76 (5)

f. To convert Rappaport Units to Boehringer milliunits:

Boehringer milliunits = (46.190) Rappaport Units − 220 (6)

This relationship is depicted graphically in Figure 3.

These correlations, either in the form of the equations or the regression curves, may be used to effect conversions among the three types of units.

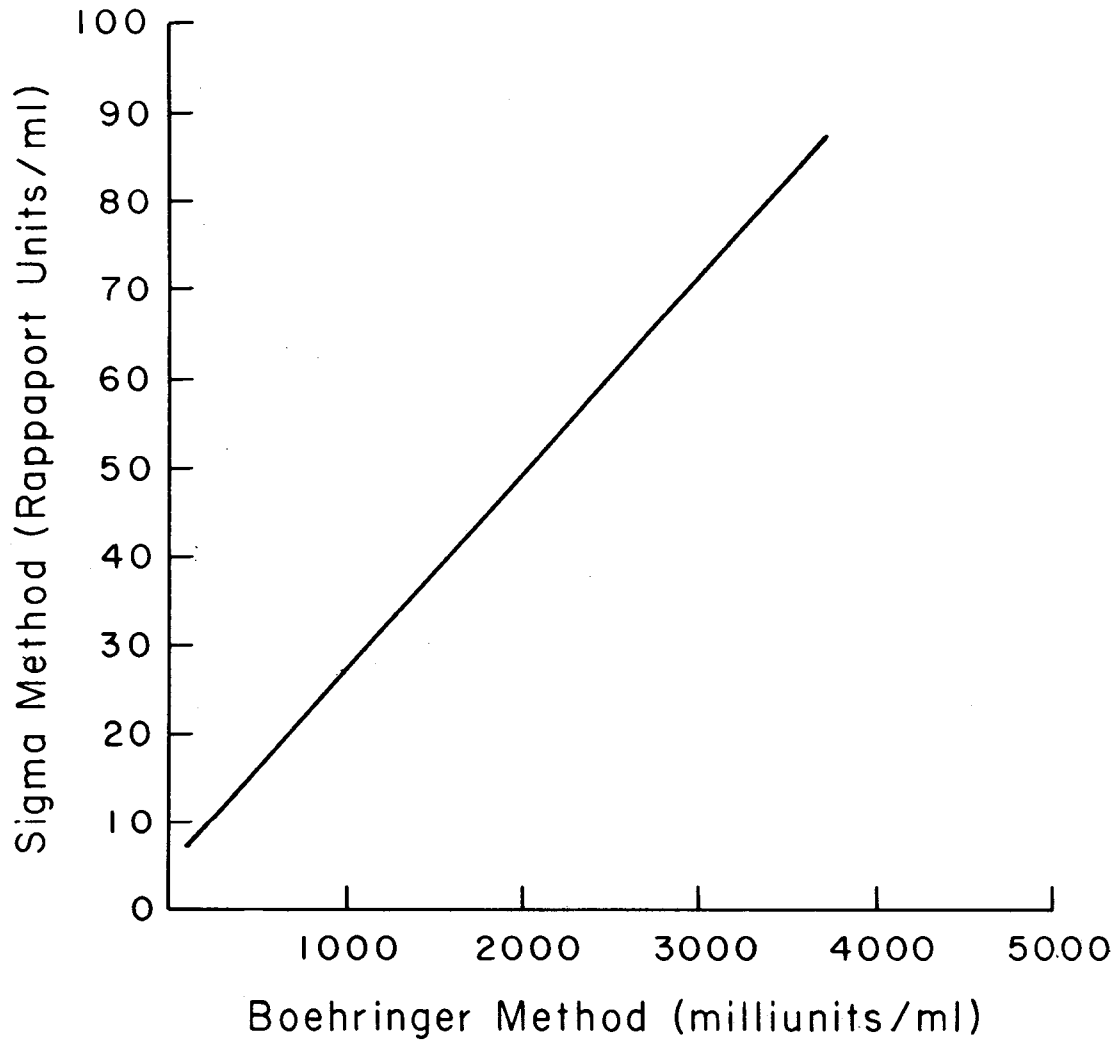

FIGURE 3. Nomogram for the interconversion of cholinesterase activity values: Boehringer milliunits—Rappaport units.

Thus, in a hypothetical case, an aerial applicator pilot could have a pre-season CHE value of 7.82 μmoles/min/ml by the pH-Stat method. Thirty days into the spraying season, a second CHE assay by the Boehringer method might provide a value of 2532 BMU, and a third assay, after an additional 30-day period, might indicate a value of 47 Rappaport units when performed by the Sigma method. All of these values are within the normal range for each of the individual methods and no single value, taken by itself, is likely to give the examining physician particular cause for concern.

However, 2532 BMU convert to 5.59 μmoles/min/ml and 47 Rappaport units convert to 4.62 μmoles/min/ml. These figures represent CHE activity losses of 29 and 41 percent respectively, when compared to the pre-season value, and indicate the progressive absorption of a cholinesterase inhibitor over the 2-month period.

The preceding example illustrates not only the practical value of the conversion capability derived from this study, since the existence of CHE inhibition would otherwise have gone undetected, but also re-emphasizes the importance of the pre-season CHE test.

REFERENCES

1. Stedman, E., Stedman, E., and Easson, L. H.: Choline-Esterase: Enzyme Present in Blood-Serum of Horse. *Biochem. J.*, 26:2056, 1932.
2. Glick, D.: Properties of Choline Esterase in Human Serum. *Biochem. J.*, 31:521, 1937.
3. Nabb, Dale P., and Whitfield, Florence: Determination of Cholinesterase by an Automated pH Stat Method. *Arch. Environ. Health*, 15:147, 1967.
4. Ellman, George L., Courtney, K. Diane, Andress, Valentino, Jr., and Featherstone, Robert M.: A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol.*, 7:88, 1961.
5. Ellman, George L.: Tissue Sulfhydryl Groups. *Arch. Biochem. Biophys.*, 82:70, 1959.
6. Koelle, George B.: The Histochemical Differentiation of Types of Cholinesterases and Their Localizations in Tissues of the Cat. *J. Pharmacol. Exp. Ther.*, 100:158, 1950.
7. Garry, Phil J. and Routh, Joseph I.: A Micro Method for Serum Cholinesterase. *Clin. Chem.*, 11:91, 1965.
8. Levine, Jacob B., Scheidt, Robert A. and Nelson, Vernon A.: An Automated Micro Determination of Serum Cholinesterase. Technicon Symposia 1965, Automation in Analytical Chemistry, Leonard T. Skeggs, Jr., Ed., Mediad Inc., New York, 582, 1966.
9. Humiston, Charles G. and Wright, George J.: A New Automated Method for the Determination of Cholinesterase Activity. *Clin. Chem.*, 11: 802, 1965.
10. Rappaport, F., Fischl, J. and Pinto, N.: An Improved Method for the Estimation of Cholinesterase Activity in Serum. *Clin. Chem. Acta*, 4:227, 1959.
11. Reinhold, John G., Tourigny, Licia Gambescia and Yonan, Virginia L.: Measurement of Serum Cholinesterase Activity. *Am. J. Clin. Pathol.*, 23:645, 1953.
12. Caraway, Wendell T.: Photometric Determination of Serum Cholinesterase Activity. *Am. J. Clin. Pathol.*, 26:945, 1956.