A Simple Method for Cholinesterase Determination Using an Expanded Scale pH Meter[1]

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INTRODUCTION

The growing interest in monitoring blood cholinesterase activities of workers exposed to pesticides or other cholinesterase inhibitors has brought a need for a simple method for determining the enzyme activity in red blood cells and serum which requires the minimum of standard laboratory equipment, expense and critical manipulations and techniques. Such a method would allow small laboratories to perform the analyses.

The method of MICHEL (1949) for cholinesterase analysis fulfills many of these requirements, but enzyme activity is expressed as Δ pH per milliliter per hour--units which have no biochemical meaning. To make quantitatively meaningful measurements, TAMMELIN and LOW (1957) modified the Michel method to give results in μmoles of acetic acid per milliliter per minute. Unfortunately, the modifications require expensive specialized equipment.

The pH Stat method of NABB and WHITFIELD (1967) monitors the production of acetic acid by automatic titration with standardized sodium hydroxide. The amount of titrant used is recorded and the results are reported in μmoles of acetic acid per minute per milliliter of sample. The equipment, however, may be prohibitively expensive for small laboratories and is not standard laboratory apparatus.

COURVILLE and LEDINGTON (1951) devised an electrometric method for serum esterase analysis in 1950, based on a pH change in a carbonate-bicarbonate solution.

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The esterase activities were expressed in units of seconds. No work was done to relate this measurement of time to biochemically meaningful units. Only serum esterases were analyzed with this method. No work followed for red blood cell cholinesterase analysis.

The method described here is a hybrid of the Michel, the Courville-Ledington and pH Stat methods. It utilizes the characteristics of a carbonate-bicarbonate solution which has maximum buffering capacities at pH 6.46 and pH 10.30 (their respective pKa's) and a region of minimum buffering capacity between pH 8.70 and pH 8.10 (± 0.3 pH of the calculated average of the pKa's). Though actually sigmoidal, a reasonably linear relationship can be attained in this region between the amount of acid formed by enzymatic hydrolysis of acetylcholine and the pH change in a defined amount of carbonate-bicarbonate solution. The carbonate-bicarbonate solution can be titrated with standardized hydrochloric acid in this chosen pH region for a conversion factor that relates Δ pH units per minute per milliliter of sample to μmoles H^+ per minute per milliliter of sample.

The use of micropipettes permits cholinesterase determinations on micro amounts of blood. Work in this laboratory has demonstrated that accurate, heparinized disposable glass micropipettes can be used both to collect blood and to determine the cholinesterase activities in red blood cells, plasma and whole blood.

MATERIALS

A. Apparatus

1. An expanded scale pH meter with output connections for a recorder (e.g. Corning Model 12 expanded scale pH meter, or equivalant).

2. Single element combination pH electrode, (preferably the micro electrode type).

3. Recorder, 100 mv, (preferably with variable speed and attenuation capabilities; e.g. Texas Instrument Servo-Riter II or equivalent).

4. Water bath set at 27°C.

5. Hematocrit centrifuge (e.g. International Centrifuge Model M.B. or equivalant)

6. Vortex Mixer.

7. Magnet stirrer fitted with a styrofoam shim such

as the bottom of a styrofoam drinking cup. The shim insulates the vessels atop the stirrer from undue warming by the stirrer's motor.

8. Teflon coated stirring magnets (1.5 cm X 0.5 cm. dia.)

9. Opticlear vials; 16 ml capacity (o.d. = 2.5 cm., height = 5.2 cm.)

10. Disposable constant bore glass micropipettes, calibrated for 100 µl with ± ½ percent accuracy from the Dade Division of American Hospital Supply Co., P.O. Box 672, Miami, Florida.

11. Crito-Seal or Dade's Miniseal hematocrit sealing clay or equivalent.

12. Tungsten carbide tipped glass marking pencil.

13. Precision rule with mm markings.

14. Tuberculin syringe, 1.0 ml with a piece of rubber tubing attached to its tip to accomodate the ends of micropipettes.

15. Tuberculin syringe, 5.0 ml with a piece of rubber tubing attached to its tip to accomodate micropipettes.

16. Tuberculin syringe, 0.5 ml with a #18 gauge hypodermic needle for the delivery of substrate solution.

17. Tuberculin syringe, 1.0 ml with a #18 gauge hypodermic needle for the delivery of substrate solution.

B. Solutions and Reagents

1. 0.11N Acetylcholine perchlorate (1.346 gm diluted to 50.0 ml with H_2O).

2. 0.01 % Saponin solution in water adjusted to pH 8.5.

3. 0.01 M Carbonate-bicarbonate in 0.9 % saline solution. About 450 ml of distilled water is added to 0.826 gm K_2CO_3 1½ H_2O and 4.5 gm NaCl in a beaker and adjusted to pH 8.75 with O.O1N-HCl solution. The adjusted solution is transferred to a 500 ml volumetric flask and diluted to volume. The final pH should be very close to pH 8.75.

4. Standardized 0.01M - HCl solution.

METHOD

A. Calibration of Carbonate-Bicarbonate Solution

To correlate the amount of H^+ produced from the hydrolysis of the substrate, acetylcholine perchlorate, with the change in pH of the dilute carbonate-bicarbonate solution at minimum buffering capacity, a conversion factor is used. This factor is in turn derived from the titration of the carbonate-bicarbonate solution with standardized HCI. A sufficiently large volume of carbonate-bicarbonate solution is used so that an accurate titration volume of HCI solution can be measured. Since the procedure herein described employs 4.25 ml of carbonate-bicarbonate solution for the determination of cholinesterase activity, a volume of the carbonate-bicarbonate solution that is twice as large (8.5 ml) is conveniently used in the calibration.

An 8.5 ml aliquot of the prepared carbonate-bicarbonate solution is transferred into an opticlear vial and brought to 27°C in a water bath. Then with stirring, the solution is titrated with standardized 0.01 M-HCI solution and the pH recorded after each 0.01 ml addition of titrant. The results of duplicate determinations are graphed to determine the acid-base characterixtic of the carbonate-bicarbonate solution over the chosen pH region (pH 8.70 to pH 8.10). From these data a conversion factor is calculated. This factor correlates the amount of acid required to cause a 1 pH unit change in 8.5 ml of carbonate-bicarbonate solution. Thus, if 0.5 ml of 0.01 M-HCI solution is required to change the pH of 8.5 ml of carbonate-bicarbonate solution from 8.60 to 8.10, then the conversion factor would be:

$$\frac{(0.01M - HCI)\ (0.5\ ml)\ (1 \times 10^{-3}\ liter/ml)}{pH\ (8.60 - 8.10)} =$$

$$\frac{5 \times 10^{-6}\ moles}{(0.50)\ pH\ unit} = 10\ \mu moles$$

For 4.25 ml of carbonate-bicarbonate solution, the factor would be one half of 10 µmoles or 5 μmH^+/pH unit.

The conversion factor can also be expressed in µmoles H^+/ml of carbonate-bicarbonate/pH unit. Such an expression would permit the use of larger or smaller volumes of carbonate-bicarbonate solution in the analyses as a matter of convenience. One note of caution should be exercised, however, when alternative volumes of the carbonate-bicarbonate solution are used. The volume of substrate added to the esterase-carbonate-bicarbonate solution before the

measurement of enzyme activity must also be changed so that the final substrate concentration is the same for the particular determination (i.e. plasma or RBC).

B. Preparation of Sample

Heparinized micropipettes may be used to draw blood directly from the earlobe or finger tips. Alternatively, blood can be drawn from the arm with heparinized vacutainers from which micropipettes can be filled. Approximately 100 µl of whole blood are drawn into 100 µl micropipettes which are then sealed with hematocrit sealing clay. Since most hematocrit centrifuge heads can accomodate tubes only up to 80 mm, the micropipettes are cut back to 78 mm, a length which more than accomodates a volume of 100 µl, prior to use.

After centrifugation at 11,500 rpm for 5 minutes, the lengths of tube containing the RBC and the total blood volumes are recorded. Knowing the length of the micropipette that represents 100 µl (from the end of the tube to the calibrated mark), the hematocrit and the total volume of blood contained in the tube are calculated. These volumes are used later for the calculations of cholinesterase activities.

For separate plasma and RBC cholinesterase analyses the tube is scratched with a tungsten carbide-tipped pencil and broken at the ends of the tube to remove the hematocrit sealing clay and at the RBC-plasma interface to divide the pipette in preparation for separate plasma and RBC cholinesterase determinations. The RBC fraction is washed from the tube into an opticlear vial with 0.5 ml of 0.01 % saponin solution from 1.0 ml tuberculin syringe and then with 4.25 ml of carbonate-bicarbonate solution from the 5.0 ml tuberculin syringe. The RBC hemolysate is then mixed thoroughly with vortex mixer. The plasma fraction is simply washed from the tube with only 4.25 ml of carbonate-bicarbonate solution into an opticlear vial and the solution mixed thoroughly.

For whole blood cholinesterase determinations, only the sealed ends of the micropipette are scratched and removed. The contents are washed into an opticlear vial with 0.5 ml of 0.01 % saponin solution and then with 4.25 ml of carbonate-bicarbonate solution. The hemolysate is mixed thoroughly. All fractions are then brought to temperature in the 27^{o}C water bath.

C. Determination of Enzyme Activity

A modest amount of non-enzymatic hydrolysis of the substrate may contribute to the measured enzyme activity and should be subtracted to give the true activity.

Nonenzymatic hydrolysis should be measured for ten minutes each day, using a volume of distilled water in place of the enzyme sample. Although the normal rate is relatively low, the nonenzymatic hydrolysis may be as high as 10 % of a measured plasma cholinesterase activity. In RBC and whole blood enzyme determinations, the nonenzymatic hydrolysis rate is an insignificant factor.

While stirring with a teflon coated magnet at a fairly high speed, a few seconds are allowed the pH electrode to equilibrate in the solution. The pH meter is set on the expanded scale and the recorder is activated to depict the pH change with time.

For whole blood or RBC cholinesterase determinations, 0.1 ml of acetylcholine perchlorate solution is added to the reaction mixture from a calibrated tuberculin syringe. For plasma cholinesterase determinations, 0.6 ml of acetylcholine perchlorate solution is added. (The pH of the sample mixture should be about pH 8.6 before the addition of substrate). Hydrolysis is allowed to proceed for 2-3 minutes (enough for an accurate measurement of a pH change).

D. Calculations of Cholinesterase Activity

From a graph of an enzyme hydrolysis reaction, a time and pH interval are chosen for calculating enzyme activity.

Cholinesterase activity in μmoles/min/ml) =

$$\left[\frac{(pH_1 - pH_2)}{({}^{t}pH_1 - pH_2)} - K\right]\left[C.F.\right]\left[\frac{1}{Vol._{smpl}}\right]$$

Where C.F. = Conversion factor as determined by titration of the carbonate-bicarbonate solution with standardized 0.01M-MCl

K = Rate of nonenzymatic hydrolysis of the substrate in the carbonate-bicarbonate solution.

${}^{t}pH_1 - pH_2$ = the time interval required for the hydrolysis over the chosen pH_1 to pH_2 interval

Vol_{smpl} = Volume of sample used as determined by proportional measurements of micropipette lengths.

RESULTS

A number of cholinesterase determinations were performed on blood samples available to this laboratory. The pH Stat method of NABB and WHITFIELD (1967) as well as the method herein proposed (which will be referred to as the pH meter method) were used.

Regression analyses of plasma cholinesterase and whole blood cholinesterase results determined by the two methods are depicted in Figures 1 and 2 respectively. Regression analysis (Fig. 1) indicates that plasma cholinesterase activities by the pH meter method are higher than those by the pH Stat method. Results of whole blood cholinesterase analyses by the pH meter method are only slightly lower than by the pH Stat method (Fig. 2).

A limited number of tests were made using micropipettes to withdraw blood from 4 volunteers. (Blood samples were drawn from each volunteer on 2 different days). The filled micropipettes were kept under refrigeration for 2 weeks before analysis. Plasma and RBC cholinesterase analyses were performed on the blood samples using both the pH meter method and a micropipette modification (manuscript in preparation) of the pH Stat method of NABB and WHITFIELD (1967). These results are shown on Table 1. Cholinesterase results by the pH meter method were generally lower than results by the pH Stat method, a finding which may reflect on the use of older blood samples. RBC cholinesterase results differ only slightly by the two methods.

Enzyme inhibition tests were made using pooled human serum, (provided by an Environmental Protection Agency laboratory) and carbaryl (a carbamate) as the enzyme inhibitor. The cholinesterase activity of 0.15 ml of the serum was determined by the pH meter method. The same amount of serum plus 5.0 µg of carbaryl resulted in a 50 % decrease in cholinesterase activity. With the same amount of serum and 2.5 µg of carbaryl, the cholinesterase activity was reduced by 40 %. Similar tests with the same human serum and the same amounts of carbaryl gave identical results with the pH Stat method.

DISCUSSION

Recorder graphs of the cholinesterase hydrolysis of acetylcholine may at times appear non-linear especially in the region of pH 8.2 or lower. The non-linearity may result from the use of older blood and may be quite noticeable in RBC and whole blood cholinesterase

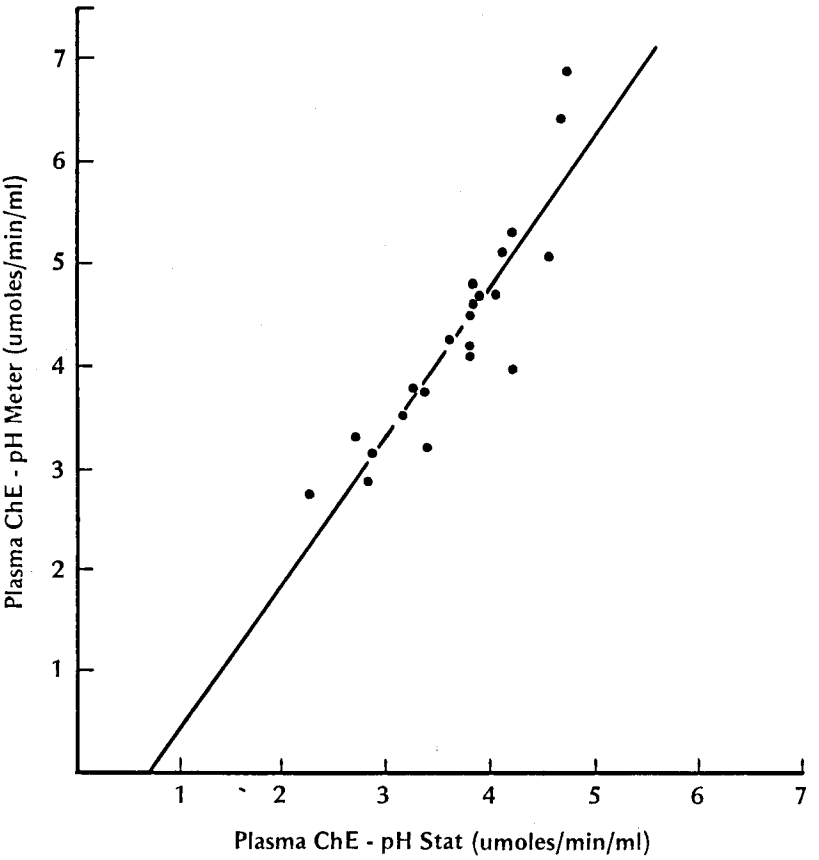

Fig. 1 Correlation between plasma cholinesterase activity when measured by pH meter and pH Stat. Regression line equation is

$$Y = -1.03 + 1.45X.$$

determinations. Generally, plasma cholinesterase determinations showed little or no non-linearity in the pH vs. time recorder graphs. The region of choice for calculating cholinesterase activity is between pH 8.7 and pH 8.3 where non-linearity is generally minimal.

The activities of both plasma and RBC cholinesterase under various pH conditions have been studied by EPA's research laboratory. These studies showed that plasma cholinesterase activity is optimal at pH 8.6. For RBC cholinesterase, optimal activity peaks broadly at pH 8.0 with 95 % of the activity between pH 7.6 and pH 8.4. The proposed method for cholinesterase analysis

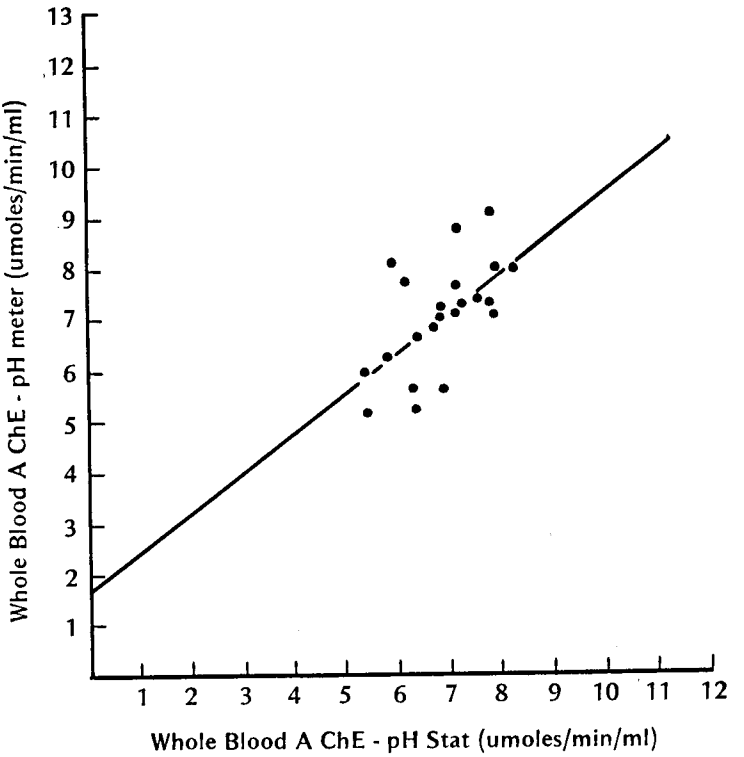

Fig. 2 Correlation between whole blood cholinesterase activity when measured by the pH meter and pH Stat. Regression line equation is

$$Y = 1.70 + 0.78X.$$

is well within this region of optimal pH conditions for both plasma and RBC cholinesterase.

With fresh substrate solutions of acetylcholine perchlorate, the nonenzymatic hydrolysis is minimal and too small to enter into calculations. Older substrate solutions, however, tend to lower the pH of the sample carbonate-bicarbonate mixtures upon their addition, and may therefore necessitate consideration in the calculations for enzyme activity. It is best, therefore, not to prepare more than a 2 or 3 day supply of substrate solution.

The titration of the carbonate-bicarbonate solution daily to establish the conversion factor is a time consuming step. When cholinesterase analyses are performed on 2 or 3 consecutive days, an alternate

TABLE I

Acetylcholinesterase Activities by Two Methods Using Micropipettes

Sample	pH Stat		pH Meter	
	Plasma AChE	RBC AChE	Plasma AChE	RBC AChE
C517	3.73	13.62	2.21	12.07
H517	5.56	14.13	5.44	11.90
L517	5.04	13.10	4.43	13.51
W517	4.43	13.50	4.55	13.11
C521	3.30	14.75	3.30	14.89
H521	7.54	14.62	5.20	14.29
L521	4.02	15.60	3.32	13.46
W521	4.52	15.63	3.52	15.52

In μmoles/min/ml,

Linear regression equations from the above data are:

Plasma AChE: $Y = 0.99 + 0.63X$

RBC AChE: $Y = 2.29 + 0.79X$

Where Y = results by the pH meter method,

X = results by the pH Stat method.

procedure may be adopted in lieu of titration. Rehydrated serum from a single lot can be used as a "secondary standard" from which a calculated conversion factor can be derived.

Lyophilized pooled human serum is commercially available from such distributors as Boehringer Mannheim Corporation. The cholinesterase activity of the rehydrated serum can be determined by using the carbonate-bicarbonate solution which has been calibrated against standard HCl. The serum, now with an assayed cholinesterase activity, can be kept frozen for re-use in calibrating the carbonate-bicarbonate solution the following day. No change in enzyme activity has been experienced when the reconstituted

serum has been kept frozen for over two weeks. A conversion factor calculated from use of the thawed serum is almost identical to that derived from the titration of the carbonate-bicarbonate solution with standard HCl The use of a control serum also normalizes enzyme determinations from day to day; thus it serves also as a reference.

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