The Clinical Significance of Erythrocyte Cholinesterase Titers

I. A Method Suitable for Routine Clinical Use, and the Distribution of Normal Values

By Jean Captain Sabine

Evidence has been published that the cholinesterase titer of erythrocytes in the peripheral blood is an indication of the activity of the bone marrow. Additional data, to be presented subsequently in Part II of this paper, substantiate this view and further indicate that under many circumstances the cholinesterase titer may be more informative than the reticulocyte count.

The procedure to be described can be carried out by any competent technician. One person can complete the determinations on six samples in about two hours, on twelve in about three hours.

The amount of blood required can be obtained by finger- or heelprick, provided a micro or semi-micro hematocrit apparatus is available.

The colorimetric method of Hestrin for the determination of acetylcholine and other carboxylic acid derivatives, based on the reaction of acetylcholine with hydroxylamine to form acethydroxamic acid which forms a colored complex with ferric ions, has been adapted for cholinesterase determinations on blood.

Reagents 4 and 8–11, and steps 12–14 and 17 of the Procedure are his.

Our method is similar to one published by Fleisher and Pope in 1954, also based on the method of Hestrin.

Reagents

Figures in parentheses indicate the amounts of each reagent used in making 12 determinations.

1. Redistilled water for reagents 2 to 6.
2. Gelatin (bacteriologic) 0.3 per cent, made up freshly each day (12 ml.).
3. Phosphate buffer, 0.67M, pH 7.0 (Na2HPO4 + KH2PO4) (25 ml.).
4. Sodium acetate, 0.001N, adjusted to approximately pH 4.5 with HCl (used in making reagent 5).
5. Acetylcholine iodide, 30 mg./ml. in 0.001N Na acetate; or acetylcholine bromide, 24.8 mg./ml. (1 ml.)
6. Quinidine sulfate, 0.2 per cent (1 ml.).
7. Trichloracetic acid, 20 per cent (12 ml.).
8. Hydroxylamine hydrochloride, 2M (24 ml.).
9. Sodium hydroxide, 3.5N (24 ml.).
10. Hydrochloric acid, 1 part conc. HCl + 2 parts H₂O (24 ml.).
11. Ferric chloride, 20 per cent, in 0.1N HCl (24 ml.).

PROCEDURE

1. Make up gelatin (reagent 2). Cool to room temperature before using.
2. Collect blood (minimum 0.15 ml.) in a small stoppered vessel containing a few grains of dry heparin.
3. Deliver 1.0 ml. gelatin into 10 ml. Erlenmeyer flasks with ground stoppers, one for each CHE determination and two for standards.
4. Deliver 30 μ (0.030 ml.) whole blood into the gelatin and rinse back until pipet is clear. Deliver 30 μ gelatin into each of the standards.
5. Place flasks in waterbath at 37 C.
6. Make up “reaction mixture”:
   - 25 ml. 0.67M phosphate buffer
   - 1 ml. acetylcholine
   - 1 ml. quinidine
7. Start hematocrits.
8. Starting a stopwatch at the same time, add 2.0 ml. reaction mixture to flask no. 1, and at 1 min. intervals to each successive flask. If there are more than 12 flasks, see Notes.
9. Prepare ice-water bath, and set up filtering tubes and aliquoting pipets (2 ml. volumetric, one for each filtrate).
10. At 30 min. add 1.0 ml. 20 per cent trichloracetic acid to flask no. 1, shake and filter immediately. At 1 min. intervals add acid to each successive flask and filter.
11. As soon as possible (and never more than 15 min. after addition of trichloracetic acid), transfer a 2.0 ml. aliquot of each filtrate to a second tube, stopper and place in ice-water.
12. Combine hydroxylamine and NaOH in equal volumes.
13. Remove a tube from the ice-water and add 4 ml. alkaline hydroxylamine, starting a stopwatch at the same time.
14. After 2 min. add 2.0 ml. HCl, and then 2.0 ml. FeCl₃.
15. Hydroxylamine can be added to aliquot no. 2 at this time.*
16. Pour the contents of the tube prepared in step 14 back and forth slowly from this tube to a colorimeter tube until most of the bubbles are gone; then tap tube with fingernail. Be sure to end up in a colorimeter tube.
17. Read at 540 mμ in a Coleman Junior Spectrophotometer or other suitable instrument.

NOTES ON METHOD

1. Standardization. Determinations on varying concentrations of acetylcholine (ACh), processed exactly as described under Procedure, gave a good straight-line relation between concentration of ACh and optical density from D = 0.065 (the intercept) to D = 0.85. From the slope a factor is calculated by which change in density (ΔD) can be converted directly into micromols (μM) ACh hydrolyzed in the original 3.03 ml. reaction volume. Our factor is 15.6; this, divided by 30, gives the factor for μM/min. (0.520).

* Steps 16 and 17 can usually be completed within 2 min., permitting addition of HCl to aliquot no. 2 on schedule. A delay up to 3 min. does not cause significant errors.
The standard error of the method as we use it, resulting from the combined errors of the cholinesterase determinations (±0.95 per cent) and the microhematocrit method (±0.49 per cent) is ±1.07 per cent. Since the desired figure was the standard error of a ratio, this was computed as the standard error of the difference between the logs of the two means.

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2. Specimen set of data.

<table>
<thead>
<tr>
<th>No.</th>
<th>T</th>
<th>D</th>
<th>Log 1/T</th>
<th>ΔD</th>
<th>t</th>
<th>VPC</th>
<th>Δ RBC</th>
<th>Rate</th>
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<td>0.477</td>
<td>0.322</td>
<td>0.321</td>
<td>0.312</td>
<td>30</td>
<td>51.8</td>
<td>15.6</td>
<td>10.4</td>
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<td>2</td>
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<td>0.323</td>
<td>0.323</td>
<td>0.310</td>
<td>30</td>
<td>51.8</td>
<td>15.6</td>
<td>10.3</td>
</tr>
<tr>
<td>S</td>
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<td>0.634</td>
<td>0.634</td>
<td></td>
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<tr>
<td>s</td>
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<td>0.632 0.633</td>
<td></td>
<td>30</td>
<td></td>
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</tr>
</tbody>
</table>

Numbers 1 and 2 were duplicate determinations on the same blood sample. The other two sets of data (S) are the two standards. T is the transmission and D the density as read from the scale of the spectrophotometer. Log 1/T is read from the slide rule to check the reading, which is difficult at the higher end of the scale. The densities of nos. 1 and 2 taken from the log 1/T column are subtracted from the D of the standards, multiplied by the factor 0.520, and divided by the volume of cells (0.0156 ml.) to give the rate as μM hydrolyzed per minute per ml. of cells.

3. Course of reaction. The rate is constant until ΔD exceeds 0.35, when it begins to fall off with time. In very few conditions are the titers high enough to exceed this limit. These are known to include pernicious anemia during response to therapy, recovery from hemorrhage, and hemolytic icterus. In such cases if anemia is not profound, it is well to make the determination with 20 X of blood instead of 30 X.

4. Standard error. Fifteen simultaneous determinations on the same sample of blood gave a mean ΔD of 0.2738. The standard error, ±√Σ(ΔD/(n - 1)), was ±0.0026, or ±0.95 per cent of the mean value. Ten of the values were 0.273 or 0.274.*

5. Side-reactions. Trichloracetic acid (TCA) reacts with ACh at room temperature, but not perceptibly at 0°C, to give a lower optical density in the last step. Filtrates should be aliquoted as soon as possible and always within 15 min. of addition of TCA. The aliquots can be left in the ice-water bath as long as three hours. Chilling the filtrates instead of the aliquots does not give uniform results.

There is also a side-reaction at the hydroxylamine stage (step 13) which results in increased density. Whether or not TCA is present, the density is decreased if a delay occurs between addition of HCl and FeCl₃ (step 14), and between this step and reading. Since all of these sources of error could be controlled by a schedule which is quite convenient and permits a break at two stages, no search was made for a more satisfactory agent to precipitate the proteins.

6. Cleaning of glassware. It is essential that glassware be thoroughly clean, not only to ensure accuracy of volumetric measurements but to avoid accidental poisoning of the enzyme. The detergent Haemo-sol (Meinecke and Co., New York) is practically devoid of anticholinesterase activity and has proved equal to dichromate-sulfuric acid as a cleaning agent. Its use obviates the excessive rinsing required when the latter agent is used.

Notes on Procedure, Reagents and Equipment

1) Procedure: Paragraph numbers refer to the steps of the procedure.

2. Blood samples. Either capillary or venous blood can be used. Collection of capillary blood is facilitated by applying a thin coat of sterile white vaseline over the fingertip or heel before puncturing. Heparin is the anticoagulant of choice for obtaining accurate hematocrits on small samples. Hematocrit determinations must be made promptly but the remainder of the blood can be preserved as long as four days by adding a few crystals of sodium citrate and covering the blood with light mineral oil to nearly fill the vial.

* The standard error of the method as we use it, resulting from the combined errors of the cholinesterase determinations (±0.95 per cent) and the microhematocrit method (±0.49 per cent) is ±1.07 per cent. Since the desired figure was the standard error of a ratio, this was computed as the standard error of the difference between the logs of the two means.
4. It has been demonstrated that the gelatin-blood mixture can be allow to stand, stoppered, at room temperature (72 F.) for two hours.

8. It is essential that aliquots of the filtrates (steps 10 and 11) be placed in the ice-water bath within 15 min. of the addition of trichloracetic acid (TCA). This must be planned for in the timing of step 8, if one person is working alone.

10. TCA can be blown from the pipette until most of it has been expelled, then the pipette drained at the surface in the usual manner.

11. Aliquots can be left in ice-water up to three hours if desired. They cannot be left overnight.

16. Bubbles well above the mid-depth of the solution do no harm, since the photocell “sees” only below the 5 ml. level.

2) Reagents: Paragraph numbers refer to the previous listing.

1. It is advisable to use water which has been redistilled in a glass still for all solutions coming into contact with the enzyme (reagents 2-6).

2. Gelatin in final concentration 0.1 per cent preserves the enzyme from spontaneous deterioration during the reaction period. It is not advisable to use water which has been redistilled in a glass still for all solutions coming into contact with the enzyme (reagents 2-6).

5. Acetylcholine in acidified acetate remains usable for several days if stored in the refrigerator. Fresh solutions should be made at least once a week. It should not be added to the buffer until just before use, since alkaline hydrolysis is appreciable at pH 7.

Time can be saved and increased accuracy achieved by lyophilizing aliquots of a solution of ACh and redissolving as needed. AChI, 1.704 Gm., or AChBr, 1.411 Gm., is placed in a beaker and dissolved in 10.0 ml. H2O. Aliquots, 1.0 ml., are delivered into 10 ml. Erlenmeyer flasks (g.g.s.) and lyophilized. The flasks are stoppered after greasing the stoppers lightly with white vaseline, and can be stored indefinitely at room temperature. For use, 5.0 ml. sodium acetate is added. The final solution contains 30 mg. AChI or 24.8 mg. AChBr per ml. The final concentration of ACh in step 7 is 2.67 X 10^{-4} M.

6. Quinidine is a selective inhibitor of plasma cholinesterase. Its use makes possible the determinations on red cells in whole blood instead of washed cells.

8. Hydroxylamine is preserved for many weeks if kept in the refrigerator. After addition of alkali it becomes too bubbly to use after about 30 min.

3) Equipment: All volumetric glassware should be of high quality (Normax or Exax).

Paragraph numbers refer to the steps in the Procedure.

4. Lambda pipettes (Northwestern or Misco) permit very accurate measurement of small volumes of blood. A control syringe (Misco) makes them very easy to use.

7. An accurate, inexpensive, and convenient semi-micro hematocrit apparatus was described in a previous publication. It requires about 0.1 ml. of blood.

9. Filtering tubes are ordinary test tubes. Small chemical funnels (25 mm. diameter) are supported by the tubes. Whatman filter paper, 7 cm., No. 42, 1, or 2 are all satisfactory.

16. Satisfactory and inexpensive colorimeter tubes can be obtained by examining a batch of Kimble bacteriologic tubes (Aloe No. 78140). Acceptable tubes all give precisely the same reading, and the reading remains constant when the tube is rotated in the beam. A suitable

* Fleisher and Pope state that ACh solutions in acidified acetate are stable indefinitely in the cold. It has been our experience that although the density of the standard remains constant, the velocity of enzymic hydrolysis tends to be decreased after about a week, as compared with the rate for the same blood sample with freshly dissolved ACh.

† When ACh is dissolved in water the volume is increased. The equation is: v = 1.00425 + 0.000622x, where v is the resulting volume when x mg. is dissolved in 1.0 ml. H2O. Probably by coincidence the correction per unit weight is the same for the iodide and the bromide. A double correction has been made for the dissolving and redissolving in the procedure described.

‡ Disopropylfluorophosphate was tried out, but proved less convenient than quinidine for several reasons.
Fig. 1.—The distribution of erythrocyte cholinesterase titers of normal adults. The mean value (10.47 μM/min./ml. R.B.C.) has been arbitrarily set equal to 1.00. The height of each column indicates the per cent of individuals falling into each range. The curve represents the normal distribution, calculated with σ = ±0.095. n = 101.

The permanent solution for testing is obtained by diluting 1 part saturated K$_2$Cr$_2$O$_7$ with 10 parts water. The yield is about 70 per cent.

17. Any instrument can be used which gives a good straightline relation between concentration of ACh and optical density. Hestrin uses a Klett-Summerson photoelectric colorimeter with a no. 54 green filter.

**Distribution of Normal Values of the Cholinesterase Titer**

In figure 1 are shown the data for 101 adults, 51 men and 50 women. All values have been expressed relative to the mean (10.47 μM/min./ml. R.B.C.) arbitrarily set equal to 1.00. The height of each column represents the per cent of individuals falling within each of 11 ranges of cholinesterase titer. The curve represents the calculated normal distribution. The standard deviation, σ = ± $\sqrt{\sum(d^2)/(n-1)}$, is ±0.095. By the “X$^2$” test* the data conform well to the normal distribution.

The mean for women was slightly (2 per cent) higher than that for men. The difference is not significant by the “t” test.†

The data of figure 1 were all obtained on individuals living at over 7,000-foot altitude. Previous data obtained at sea-level are not directly comparable because a different method was used. The standard deviation was the same in both

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A series of 18 children aged three months to ten years, with normal hemograms and suffering from very minor ailments, has given a mean value 1.02 times the mean adult value with all values within the normal adult range. Although the series is small, it provides acceptable evidence that older babies and children have the same titers as adults.

Newborn babies, however, appear to present a different picture. Only seven have been available for study. These were from zero hours (cord blood) to fifteen days of age with the usual high red counts and hematocrits of newborns. The cholinesterase titers were from 0.57 to 0.75 times the adult mean value, with their own mean 0.65.

The spread of data on normal individuals is in agreement with that of 15 normal adults published by Sawitsky and co-workers in 1948. The absolute values are not comparable because the method and experimental conditions were quite different from ours. The standard deviation, calculated from their table, for cholinesterase titers expressed per unit volume of cells, is ±0.9 per cent of the mean value, the same as ours. Also in agreement with them, we have found that the titer for any normal individual remains quite constant over long periods of time.

SUMMARY

A method for determining erythrocyte cholinesterase titers on small samples of whole blood is described in detail. The method is one which requires little special equipment and can be used by any competent technician.

Data for erythrocyte cholinesterase are presented on a group of 101 normal adults, and it is shown that the observed data fit the normal distribution. Eighteen children, aged three months to ten years, all had values within the adult distribution, and the mean value was the same as that for adults. Seven newborn babies all had values at or below the lower limit for adults.

SUMMARIO IN INTERLINGUA

Es describite in detalio un metodo pro determinar titros de cholinesterase del eritrocitos per medio de parve specimen de sanguine integre. Le metodo require pane equipamento special e pote esser usate per omne competente technico.

Es presentate datos in re un gruppo de 101 adulti normal. Il es demonstrate que le datos es ben conforme al distribution normal. Dece-ocio infantes de etates de inter 3 menses e 10 annos habezha omnes valores intra le distribution del adultes. Septe neonatos habezha omnes valores al limite inferior o infra le limite inferior del valores pro adultos.

REFERENCES

ERYTHROCYTE CHOLINESTERASE TITERS. 1


6 Wilson, I. B., and Bergmann, F.: Studies on cholinesterase. VII. The active surface of acetylcholine esterase derived from effects of pH on inhibitors. J. Biol. Chem. 185: 479, 1950.


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