METHODS OF DETERMINING SPECIFIC AND NONSPECIFIC CHOLINESTERASE IN MICROVOLUMES OF BLOOD

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Recently many reports have been made of the estimation of the cholinesterase activity of the blood serum and red cells. More than 100 variations of methods of determining cholinesterase chemically in the blood have been published, in addition to a large number of articles dealing with the rate of enzyme activity in health and in desease. The considerable effort made to work out simple and sufficiently precise methods has been stimulated not only by the importance of cholinesterase as a transmitter of nervous impulses, but because of the growing importance of determining its activity in a number of clinical conditions [2,6,7,8,10,13,16,18,19,22,23,24]. It must also be remembered that cholinesterase activity may be supressed by means of various toxic substances, some of which are being increasingly applied in agriculture as insecticides containing phosphorus [17].

Unfortunately, most of the methods designed for the clinical determination of cholinesterase activity in the blood take account only of the nonspecific cholinesterase of the serum [1,4,9,11,12,14,20]. However, the necessity for obtaining blood serum makes it less easy to carry out a series of observations, both because rather large quantities of blood must be obtained by venipuncture, and because time must be spent in separating the serum. When working with small laboratory animals such as mice, rats, frogs, etc., repeated estimations of serum cholinesterase activity cannot be made, for the reasons already given.

Methods for the determination of cholinesterase in the erythrocytes of whole blood are much more complex and laborious [5,15,21].

These considerations have led us [3] to propose a comparatively simple colorimetric method for determining cholinesterase activity in a drop of blood. However, the method suffered from several defects; the color changes were not sufficiently sharp, which made it difficult to determine the end point, and it was not possible to separate the specific and nonspecific cholinesterase activities.

The purpose of the present communication is to describe a very much more precise and simple method for the differential determination of specific and nonspecific cholinesterase activities, which may be applied to a few microliters of blood. In developing the method we have attempted to stay close to the simple blood analysis which is well known in pathological laboratories.

In order to work out ways for the differential determination of the specific and nonspecific components, we studied the rate of enzymatic hydrolysis of equimolecular solutions of the different esters by the cholinesterase of the blood serum and of the erythrocytes (Table 1).

TABLE 1. Rate of Hydrolysis of Equimolecular Solutions of Choline Esters by the Cholinesterase of the Blood Serum

Ester	Time for 50% hydrolysis		Ratio of time of hydrolysis of ester to that of aceylcholine
	Mean (of 10 determinations	Variation range	
Acetylcholine	8,0	6.0-10.0	1.0
Acetylthiocholine	7.5	6.0-9.0	0.94
Butyrylcholine	4.0	3.5-4.5	0.50
Butyrylthiocholine	4.5	3.5-6.0	0.56

It can be seen from Table 1 that butyrylcholine is hydrolized by the nonspecific cholinesterase of the serum twice as rapidly as is acetylcholine; it is also known that the rate at which butyrylcholine is broken down by the specific cholinesterase of the erythrocytes is extremely slow, so that this quantity may be neglected.

We have developed three variants for the method of determination of cholinesterase activity which can be applied to comparators for droplet reactions and for reactions in test tubes and in blood count pipettes. In all three arrangements, the principle for the determination of the enzymatic activity is to determine the time required to break down 50% of the choline ester taken: for the determination of nonspecific esterase the choline used is butyrylcholine, while acetylcholine serves for the determination of specific and nonspecific esterases.

The duration of the reaction is measured from the rate of change of the color of an indicator in a standard buffer solution, caused by the liberation of acid through the hydrolysis of esters. The colorimetric comparison is made by using standard solutions whose color corresponds to the initial and final color of the reactive mixture. To mask the scarlet color of the blood, a rather concentrated solution of bromthiol blue is used, whose color changes from blue to yellow over a pH range of 7.6 to 6.0.

Reagents Require

1. Principal buffer indicator solution. Two hundred mg of bromthiol blue indicator are triturated in a mortar with 20 ml of 0.1 N solution of caustic soda. The blue solution is poured from the mortar into a measuring flask. The mortar is washed out several times with small amounts of water free from carbon dioxide, and the washings are added to a 1,000 ml measuring flask. Fifty ml of 0.1 M solution of boric acid (in a 0.1 M solution of potassium chloride) and 5 ml of a 5% saponin solution are then added. The contents of the flask are made up to the mark with distilled water free from carbon dioxide. The solution so obtained is of a saturated blue color, and has a pH of 8.4.

2. Buffer-indicator solution for preparing colorimetric standard No. 2. A mixture is made of 9.75 ml of the principal solution No. 1 with 0.25 ml of 0.1 N hydrochloric acid. The solution has a saturated yellow color and a pH of 3.5. The solution is stable.

3. Buffer-indicator solution of butyrylcholine for determining nonspecific cholinesterase. A mixture is made in a small beaker of 1.8 ml of the principal solution No. 1 with 0.2 ml of 1.57% of butyrylcholine (or 1.1% solution of butyrylcholine chloride). This solution, like solution No. 4, must be prepared not more than three hours before it is used.

4. Buffer-indicator solution of acetylcholine for determination of specific and nonspecific cholinesterase. A mixture of 1.8 ml of principal solution No. 1 with 0.2 ml of a 1% solution of acetylcholine is made in a small beaker.

To carry out the reaction in Vidalevskii test tubes and in blood count pipettes for incubation at 40°, a plastic thermostat comparator was made. It consisted of a rectangular box made of pieces of plastic stuck together, and containing a stand made of white plastic for holding test tubes or blood count pipettes.

When carrying out the reaction by the droplet method, we used the comparator which we had developed ourselves for this reaction. The principal feature of the comparator was a disc of colorless plastic 7-10 mm thick with recesses drilled in it of uniform depth and diameter. For most of the tests we used recesses having a 5 mm diameter and a depth of 8 mm, which held about 0.15 ml of fluid. They were arranged in two or three rows at an equal distance from each other (Fig. 1). The second important feature was the screen having a black and white surface. It served as a base for the comparator, and was connected with the disc mentioned above by a spring arm which enabled the relative position of the disc and screen to be changed by a rotation, so that a uniformly illuminated background was obtained. The black surface could be changed for a white by rotating the arm through 180°.

In order to enable samples to be placed in the thermostat, a construction was arranged which made it possible to move the plate containing the recesses within the plastic thermostat comparator described above (Fig. 2).

Method of carrying out the reaction. After the skin of a finger has been punctured, 20 microliters of blood is taken (the volume of one pipette for collecting hemoglobin) and it is blown into a test tube with 0.4 ml of distilled water. If necessary, the operation may be interrupted for 3-5 hours at this stage. When a determination is to be made in the recesses of the comparator for droplet reactions, by means of calibrated pipettes (calibrated to give drops of a standard size), two drops of hemolyzed blood are introduced, and the comparator is placed for 5-10 minutes in a thermostat containing water at 40°, in order to equalize the temperature. Then three drops

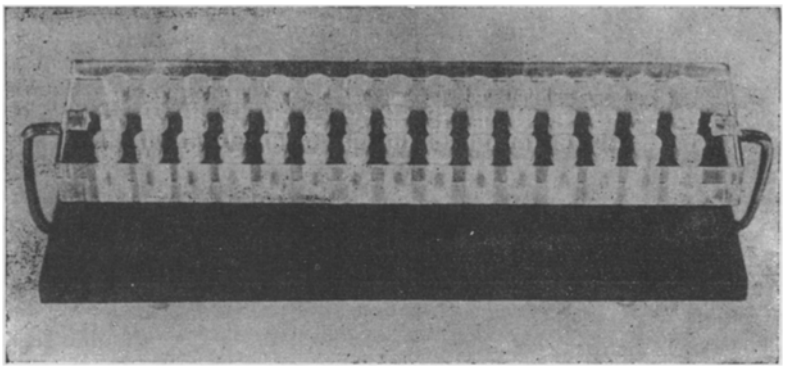

Fig. 1. Comparator for droplet reactions

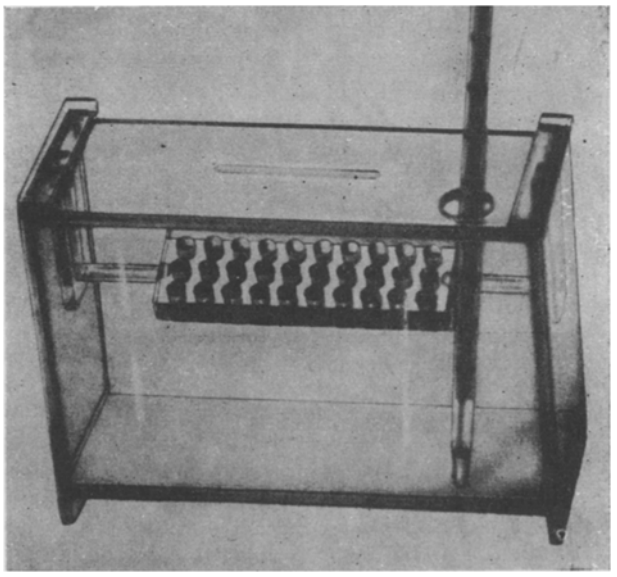

Fig. 2. Arrangement of the comparator for droplet reaction within the thermostat.

of solutions Nos. 1,2,3 and 4 are introduced into the appropriate compartments. The contents of the recesses are stirred up with fine glass rods (sealed-off capillary tubes) and the time of the onset of the reaction is noted. In this way two colorimetric standards are obtained, and two samples tested for the determination of specific and nonspecific cholinesterase. The color of the contents of the first recess, into which solution No. 1 was introduced, corresponds to the initial color of the reaction mixture (standard No. 1). The contents of No. 2 recess, into which solution No. 2 was introduced, has a pale brown color, which corresponds to the color given by a sample in which 50% of the substrate is broken down (standard No. 2). In the third and fourth recesses, to which solutions No. 3 and 4 are added, there is an enzymatic breakdown of acetyl- and butyrylcholines. The rate of change of color of these recesses away from the second standard indicates the activity of the specific and nonspecific cholinesterases in the blood under test. The time taken for the equalization of the color of the two samples (with acetyl and butyrylcholines) is found from the colorimetric standard No. 2. Colorimetric comparisons are carried out without removing the comparator from the thermostat, and are made against the white background of the plastic base plate.

When the determination is made in test tubes, the tubes are placed in the holder of a thermostat. To each tube 0.1 ml of hemolyzed blood and 0.5 ml of water is added. To equalize the temperature, the stand is placed for 5-10 minutes in a thermostat containing water at 40°. Then 0.15 ml of solutions Nos. 1,2,3 and 4 is added to each of four tubes. As a result, as in the previous method, two colorimetric standards are obtained, one of which corresponds to the original (blue-green) color, and the second to the recorded (yellow-brown) color of the reaction mixture formed after breakdown of 50% of the substrate. The contents of the tubes are carefully mixed, and the time of onset of the reaction noted. The time is taken at which equalization of the color of the samples with standard No. 2 occurs. The colorimetric comparisons are made without moving the test tubes from the thermostat, and are made against the white background of the back wall of the stand.

Calculation of cholinesterase activity. In solutions No. 3 and No. 4, choline ester is present in a concentration of 0.005 M, and in standard solution No. 2, acid is introduced at a concentration of 0.0025 M, which corresponds to a breakdown of 50% of the substrate. To compare the reaction mixtures, two volumes of hemolyzed blood are mixed with three volumes of the solutions. Because the blood is diluted 1: 50 times, and a concentration of the substrate is 0.003 M (or 1.5 micromoles per ml for 50% breakdown), the equation may be written as follows:

$$A_{ch} = \frac{1.5 \cdot 50}{T} = \frac{75}{T}.$$

where Ach is the activity of the blood cholinesterase expressed in micromoles of choline ester broken down per ml of blood per minute. In calculating the activity of the specific (A_1) and nonspecific (A_2) cholinesterase, it must be remembered that butyrylcholine is broken down by the nonspecific cholinesterase of the blood serum 100 times more actively than by the specific cholinesterase of the erythrocytes. Therefore, the value for the enzymatic activity obtained by estimation with solution No. 3 (A_3) indicates the total activity of both enzymes. In other words, A_3 (solution No. 3) = A_1 (cholinesterase erythrocytes) + A_2 (cholinesterase serum from acetylcholine).

Note (see Table 1) that a constant equal to the ratio of the activity of serum cholinesterase (as indicated by acetylcholine) to the activity of serum cholinesterase (as indicated by butyrylcholine) is approximately 0.5. Hence, the activity of the specific cholinesterase may be calculated from the following formula:

$$A_1 = A_3 - 0.5A_4,$$

where A_4 is the activity of the nonspecific cholinesterase of the blood with respect to butyrylcholine.

It must be emphasized, however, that such calculations are very arbitrary, because other kinds of esterases may also take part in the breakdown of choline esters, and there may be considerable variation in the amount of such esters between different species of animal. It is therefore more correct to express the values obtained for cholinesterase activity purely as the number of micromoles of acetyl- and butyrylcholines broken down per ml of blood per minute (or per hour).

The methods we have described were checked by testing more than 200 healthy subjects and patients, as well as several animal species. Clinically, it was possible to demonstrate pathological conditions by applying the method to cases of damage to the liver parenchyma, where cholinesterase activity attains an extremely low value.

Table 2 shows the mean values of the cholinesterase activity of the blood of some animals and man.

TABLE 2. Cholinesterase Activity of the Blood of Some Animals and Man (hydrolysis at 38°)

Blood	Time for hydrolysis to attain standard no. 2, min		Cholinesterase activity, μM/ml/min		$K = \frac{A_3}{A_4}$
	butyrylcholine (soln. 4)	acetylcholine (soln. 3)	butyrylcholine (A_3)	acetylcholine (A_4)	
Man	18,5(11—26)	16,8(10—23)	5,7	5,9	0,97
Monkey	15,8(13—19)	23,4(21—28)	6,3	4,3	1,5
Dog	33(22—44)	55,4(46—68)	3,0	1,8	1,6
Guinea pig	16,7(12—20)	33(24—40)	6,0	3,0	2,0
Rabbit*	>3 hr	48,6(37—59)	0,5	1,0	—
Mouse	21,2(14—26)	25,2(17—29)	4,7	4,0	1,2

*Because of the low concentration of the enzyme, the volume of the buffer solutions Nos. 3 and 4 was reduced by almost half in determining the cholinesterase activity in rabbit blood.

Note: The limits of variation are indicated in brackets.

In considering Table 2, it can be seen that in different human samples, and in different samples from the same animal species, cholinesterase activity, as indicated by the hydrolysis of both acetylcholine and butyrylcholine, varies within quite wide limits.

From the average figures, and particularly for the ratio of the rates of hydrolysis of butyryl- and acetylcholines of whole blood, it can be seen that there is a real difference between the cholinesterase "spectrum" of the blood of different species.

The methods described showed to particular advantage in experiments on small animals. It was then possible to follow the change in enzymatic activity from repeated blood samples taken, for example, from the tail of a mouse. An illustration may be given from the changes in cholinesterase activity following a subcutaneous injection of 0.1 mg/kg of proserine (Fig. 3).

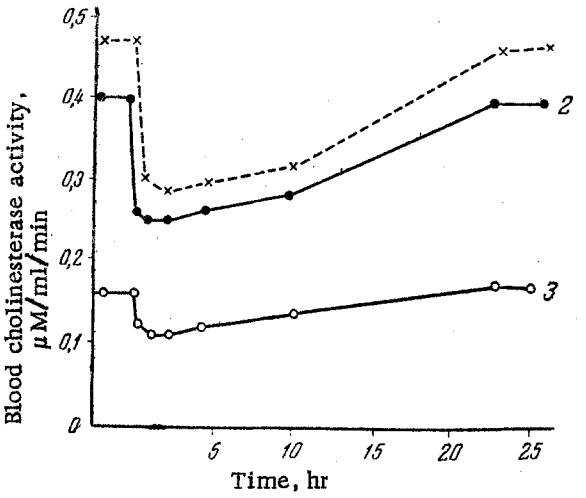

Fig. 3. Changes in cholinesterase activity in the blood of white mice which have received a subcutaneous injection of 0.1 mg/kg of proserine. 1) Rate of hydrolysis of acetylcholine (A_3); 2) rate of hydrolysis of butyrylcholine of nonspecific cholinesterase (A_4); 3) calculated rates of hydrolysis of acetylcholine by specific cholinesterase (A_1).

It is clearly seen that proserine, as a powerful but reversible inhibitor, causes a temporary suppression of both nonspecific and specific cholinesterase. By the end of the first 24 hours, the body of the mouse is completely free from proserine.

SUMMARY

A technique is described for drop colorimetric determination of the specific and nonspecific cholinesterase activities in microvolumes of blood; the method is based on changes in the hydrogen ion concentration in the reactive mixtures.

A new device makes it possible to examine a large series of samples; only a few microliters of blood are used for each test. The suggested method may also be used for repeated determination of cholinesterase activity in the blood of small laboratory animals such as mice. Mean blood cholinesterase activity values are given for various animals.

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