A SIMPLE PROCEDURE FOR THE ESTIMATION OF SERUM CHOLINESTERASE BY MEANS OF COMPARATOR WITH PHENOL RED AS INDICATOR

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For the past ten years an increasing interest in the serum cholinesterase, a non-specific enzyme which hydrolyses acetylcholine into acetic acid and choline, has become evident, and its clinical significance has recently been thoroughly reviewed by Okinaka and Yoshikawa,[9),10)] and by Vorhaus and Kark.[16)]

Several methods, different in principle, are at present available for the measurement of this enzyme: (1) gasometric procedure with Warburg's apparatus,[2)] (2) titrimetric method[3),17)] in which the acetic acid liberated from the substrate is titrated with alkali, (3) colorimetric method[5)] which measures the choline released from acetylcholine photometrically, and (4) hydrogen-ionometric procedure[1),7)] that estimates electrometrically the fall of pH in the medium caused by the liberation of acetic acid.

Gasometric method requires extreme caution and skill as well as an expensive instrument, and even the electrometric procedure which was first invented by Michel[7)] in 1949 and contributed the accumulation of a vast bulk of knowledge on serum cholinesterase necessitates special equipment, i. e., a glass electrode pH-meter that is extravagant for ordinary clinical laboratory. Titrimetric and colorimetric methods are not efficient in dealing with a great number of serum samples in a limited time, though feasible with common laboratory equipment.

In our laboratory a hydrogen-ionometric method by means of comparator with phenol red as pH indicator was developed[8),13),14)] for the approximate estimation of serum chotinesterase, and has been in routine use for three years.

A mixture of acetylcholine chloride solution and barbital glycerophosphate buffer solution is introduced in equal amount into two test tubes and used as buffered substrate. To one of these are added the serum (sample tube) and to the other distilled water in volume equal to that of serum (blank tube), and after phenol red solution is dropped, they are incubated at 37 C. At the end of one hour the enzymatic activity is stopped by eserine salicylate, and their pH is measured with the standard series of phenol red tubes in a comparator. Thus the activity of serum cholinesterase is computed in terms of the pH fall in the sample tube minus the non-enzymatic pH drop in the blank tube. The procedure is simple and requires

no special instrument. It is especially suited for and efficient in the laboratory diagnosis of hepatobiliary disturbances.

However doubt may arise about the reliability of the estimate in this procdure. Since the activity of enzyme is, in general, sensitive to the change in pH of the medium, the process at constant pH is essential for its accurate determination. Our procedure estimates, as has been stated, the pH fall in the buffered substrate instead of keeping the pH of medium unchanged throughout the process, and on this account it runs in contravention to the conventional principle of enzyme assay. Special consideration was accordingly given to the preparation of buffered substrate so as to make the hydrolysis of acetylcholine progress within the limits where cholinesterase activity is little influenced by the change in pH, and hence the drop of pH of the buffered substrate represents the amount of acetic acid liberated from the substrate, obviating the interference of non-enzymatic hydrolysis of acetylcholine as perfectly as possible.

Shortly before this paper was written an adaptation of photoelectric hydrogen-ionometry to Michel's procedure with phenol red as indicator was published by Reinhold and others.[11] Our method is advantageous over theirs in the simplicity of technic, although the estimates are in approximation.

Methods

Peagents

(1) Buffer solution. Dissolve 0.5 Gm. of sodium barbiturate and 2.0 Gm. of sodium glycerophosphate in 400 ml. of distilled water. The pH of this solution is 8.3. Add five to ten ml. of chloroform and preserve in a refrigerator. It keeps for at least two months.

(2) Acetylcholine solution. Prepare, immediately before use, the aqueous solution of Ovisot (acetylcholine chloride of Daiichi Seiyaku Co., Tokyo, contained in an ampule 0.1 Gm. in amount) in the proportion of one ampule to two ml. of distilled water.

(3) Phenol red solution. Add and pestle 0.1 Gm. of phenol red to 2.85 ml. of 0.1 N sodium hydroxide solution and 7.5 ml. of distilled water contained in an agate mortar, and make to volume with distilled water in a glass cylinder which has a mark at 250 ml.

(4) 0.01 Gm./100 ml. aqueous solution of eserine salicylate.

Procedure

Into individual test tubes (of the same size as those of the pH comparator) A (blank), B_1, B_2, ····· introduce the distilled water, the serum samples S_1, S_2, ····· and the reagents in the order given below, left to right, mix and incubate in a water bath at 37 C for an hour.

Test tubes	Buffer solution (ml.)	Distilled water (ml.)	Phenol red solution (ml.)	Acetyl-choline solution (ml.)	Serum S_1 (ml.)	Serum S_2 (ml.)
A	1.5	3.1	0.2	0.5		
B_1	1.5	3.0	0.2	0.5	0.1	
B_2	1.5	3.0	0.2	0.5		0.1
.	.	.	.	.		.
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At the end of the specified time add a drop of eserine salicylate solution to each, mix and read the pH of the tubes a, b_1, b_2, ·····(for the test tubes A, B_1, B_2, ····· respectively) in a comparator with the standard phenol red tubes. Kayagaki's (Tokyo) comparator set is employed for this purpose in our laboratory. However any sets will be available, if the amount of phenol red solution to be added to the buffered substrate is adequately adjusted.

Compute the activity of cholinesterase activity ΔpH for each sample (S_n; n= 1, 2, 3,·····) as follows.

$$\Delta\text{pH}\ (S_n) = a - b_n$$

When the initial pH for tube A, as measured immediately after its preparation, is less than 7.7, reassay with fresh reagents is required, for this indicates the staleness of either of the buffer solution or Ovisot. Serums of healthy persons give ΔpH ranging from 0.8 to 1. 1.

Before this procedure was developed, a series of experiments was carried out in the hope that the maximum reliability and accuracy of values for enzyme activity obtained in this way might be attained.

Experiment 1. To examine the buffering capacity of substrate with special reference to the pH fall of the buffer solution by acidifying. Into each of the test tubes T_1, T_2, T_3, ·····, T_{12} are introduced 1.5 ml. of buffer solution and 3.0 ml. of distilled water. The tubes T_1 to T_7 are acidifyed by the addition of 0.6, 0.5, 0.4, ···, 0.1 and 0.0 ml. of 0.1 N hydrochloric acid and those of T_8 to T_{12} alklalinized by the addition of 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of 0.1 N sodium hydroxide solution, respectively. After their volumes having been made to 5.1 ml., pH was measured with glass electrode pH-meter. All of them were acidified further by adding 0.2 ml. portions of 0.1 N acetic acid; their hydrogen ion concentration was determined in the same manner as above.

Experiment 2. To examine the influence of pH shift upon the activity of serum cholinesterase. A series of buffer solution varying in pH from 5.0 to 9.0 were prepared in the same way as in experiment 1, and cholinesterase was determined at various pH by our procedure with human blood serums as material. The values for ΔpH were expressed in percentage, assuming that the ΔpH at pH 7.7–7.6 is equal to 100 per cent for every individual serum, and these were regarded

as the activity at the mid-point of the initial and final pH. Initial pH refers to the pH of the mixture of serum and buffered substrate as determined shortly after preparation, and terminal pH to the pH of the mixture at the end of the procedure.

Experiment 3. To examine the non-enzymatic hydrolysis of substrate. To the test tubes were introduced the buffer solutions varying in pH from 6.2 to 10.0 prepared as in experlment 1, distilled water, acetylcholine chloride solution and again distilled water instead of serum, each in volumes designated in our procedure, and the initial and final pH was measured with a glass electrode pH-meter. The non-enzymatic hydrolysis of acetylcholine was evaluated by the difference between the initial and the final pH.

Experiment 4. To examine the accuracy of comparator hydrogenionometry. As soon as the final pH of blank and sample tubes has been measured in a comparator with phenol red as indicator, it was checked by glass electrode pH-meter, and the two lots of ΔpH obtained in these ways were collated to each other for eighty serum samples to see if discrepancy was appreciable.

Experiment 5. Fifteen serums were estimated for serum cholinesterase activity by Michel's method simultaneously with our procedure adapted to glass electrode pH-meter to ascertain the correlationship of two lots of ΔpH determined by these ways.

Results and Discussion

Figure 1 represents the shift of pH in our buffer solution by acidifying and alkalinizing (Experiment 1). It is apparent from the curves, which reveal the effect of the addition of hydrochloric acid and sodium hydroxide (curve AA) as well as of the further addition of acetic acid (curve BB) to our buffer solution that the pH falls exactly in proportion to the amount of the acid added in the range of test tubes from T_4 (pH 6.5) to T_7 (pH 8.0), whereas it does not necessarily parallel the intensity of acidifying or alkalinization in the tubes T_1—T_3 and T_8—T_{12}. Our buffer solution is accordingly qualified to indicate the exact quantity of acetic acid liberated from acetylcholine in so far as its final pH falls into the range over 6.5.

The activity of serum cholinesterase in relation to pH of the buffered substrate (Experiment 2) is depicted in Figure 2. Inspection of the figure reveals that the activity is kept constant, being little affected by the variation of pH, at least within the limits of pH 7.7 to 6.7. Since our buffer solution has pH 7.8 or 7.7, as measured immediately after acetylcline solution and serum are added, and seldom does it drop as low as pH 6.6 at the end of one-hour's incubation at 37 C, the ΔpH, in our method, can be estimated with little interference of pH in enzymatic activity.

Opinions differ concerning the influence of pH upon the activity of cholinesterase. Glick[4] stated that cholinesterase diminished its activity with decrease in

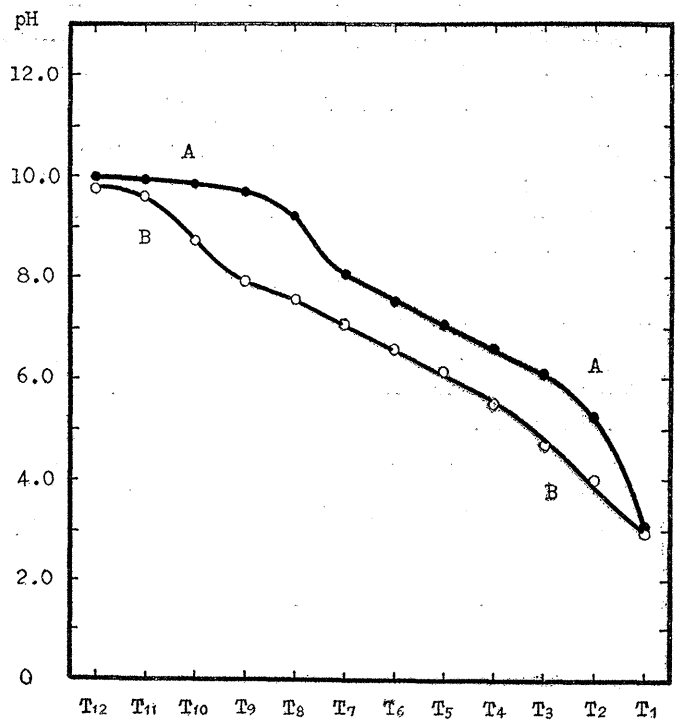

Fig. 1. Shift of pH in the buffer solution by acidifying and alkalinizing (Experiment 1).

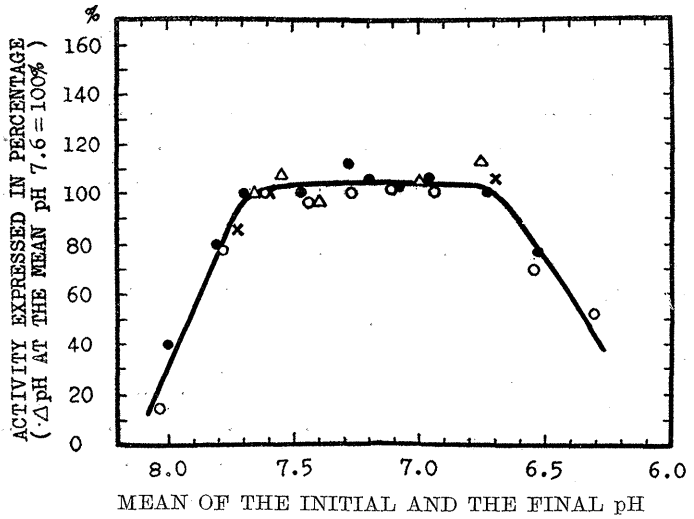

Fig. 2. The activity of serum cholinesterase in relation to pH of the buffered substrate (Experiment 2).

pH within the range of from pH 8.5 (maximum) to 6.0 (extremely enfeebled). Michel[7] established his procedure on the observation that both the barbital buffer solution and cholinesterase had their maximum capacity and activity at pH 8.0, and weakened progressively as pH fell. Sumner[12] described in his text-book, on the contrary, that the majority of workers measured the activity at pH 7.0, because detailed information on the effect of pH upon the activity of cholinesterase was hardly available, and Koelle[6] also pointed out that non-specific cholinesterase of horse serum was 1.25 times as active at pH 6.4 as at pH 8.0. Our data (Experiment 2) is in good agreement with Koelle's observation.

Non-enzymatic hydrolysis of acetylcholine (Experiment 3) is negligible at the pH below 7.2 and slightly appreciable between pH 7.2 and pH 7.7, though it becomes distinct in the region over 7.8, as clearly shown in the following figures which are picked up from the record of our experiment: pH falls are equal to 0.00, 0.25, and 0.72 for the initial pH 7.1, 7.7 and 8.2, respectively. The blank tube is therefore liable to non-enzymatic decomposition, thus giving rise to a pH reduction of approximately 0.15 during one-hour's incubation at 37C, because it remains in the pH boundary of active non-enzymatic hydrolysis throughout the procedure. The sample tube is also, of course, not free therefrom, but it is less influenced, since non-enzymatic decomposition is at work only while it passes through the pH between 7.7 and 7.2. As a corollary ΔpH may assume theoretically an unreasonably small value in case of cholinesterase-rich serum, though actually the decrement is so minute that it does not exceed 0.1, being masked by the systematic error which is inherent in the comparator hydrogen-ionometry. Non-enzymatic hydrolysis in the blank tube is not stopped by the addition of eserine, but persists throughout the time when it is allowed to stand at room temperature for hydrogen-ionometry, and its pH falls approximately 0.1 in several hours. The pH reading should accordingly be finished as soon as the test tubes are withdrawn from the water-bath. The blank tube aims to eliminate the fluctuation of ΔpH caused by the heterogenous quality of Ovisot which varies more or less with the initial pH on every determination.

Acetylcholine chloride solution of 0.03 Mol is employed in our procedure as the best substrate, since less concentrated solution causes the shortening of the range of variation of ΔpH, thus entailing the insensitivity of the determination. De la Huerga,[5] realizing that optimum concentration lies over 0.03 Mol, chose 0.045 Mol solution as substrate. Michel[7] and Alcalde[1] used 0.035 and 0.05 Mol solutions, respectively. In our opinion 0.03 Mol is most desirable, because non-enzymatic decomposition becomes more pronounced with increase in the concentration of acetylcholine and hence interferes with ΔpH.

Figure 3 reveals the reliability of comparator hydrogen-ionometry in our procedure as checked by the glass electrode pH-meter (Experiment 4).* The cor-

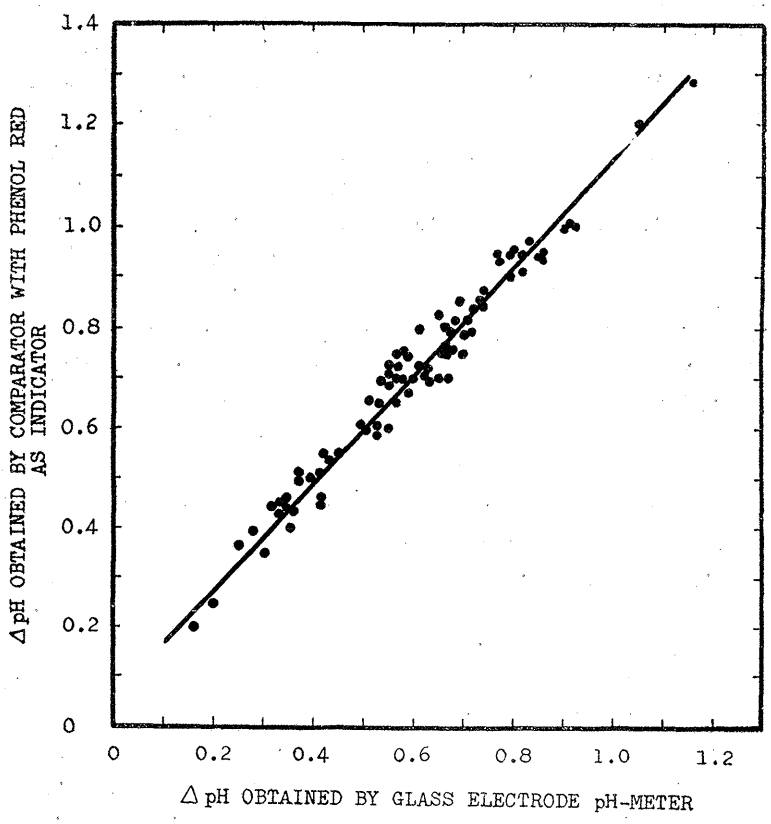

Fig. 3. The correlationship of ΔpH obtained by comparator to that by glass electrode pH-meter in our procedure.

relationship of ΔpH obtained by comparator (ΔpH (c)) to that by glass electrode pH-meter (ΔpH (g)) is expressed in the equation

$$\Delta\text{pH (c)} = 1.1 \times \Delta\text{pH (g)} + 0.05$$

That the values for ΔpH are systematically higher in the former than in the latter is attributed to the deviation of pH reading toward higher direction. The barbital glycerophosphate buffer gives too high or too low figures for pH reading when it is compared with Kayagaki's standard phenol red tubes, depending on whether its true pH (checked by glass electrode pH-meter) lies over or below 7.1. To take an example, the buffer solution of pH 8.0 is read as pH 8.2, whereas that of pH 7.1 is given exactly as 7.1. A delicate change in the shade of phenol red color

* In our experience the pH of the sample tube seldom falls below 6.8, a critical level for comparator reading with phenol red as indicator. Normal sample tubes invariably reside within the range which permits comparator reading.

arises when serum is added to the buffer solution, and this is partially responsible for the deviation of ΔpH of comparator procedure from that of electrometric technic amounting to $\pm$ 5 per cent which is perceived by the scattering of dots from the linear line depicted in this figure.

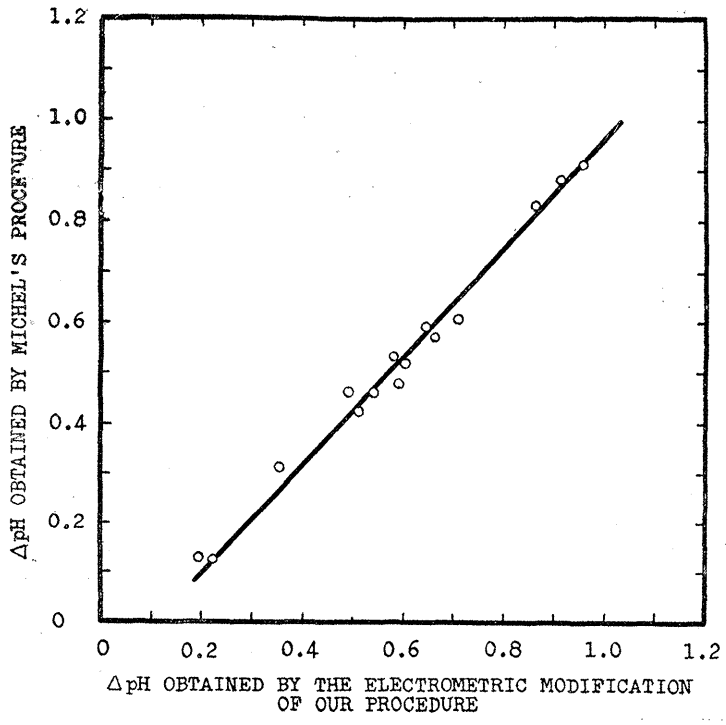

Fig. 4. The intimate correlationship of ΔpH between Michel's procedure and a modification of our method adapted to electrometric technic (Experiment 5).

Figure 4 discloses the intimate correlationship of ΔpH between Michel's procedure and a modification of our method adapted to electrometric technic (Experiment 5).

Twenty blood serums of healthy persons who had neither jaundice nor hepatic diseases in the past were determined for cholinesterase by our procedure and it was ascertained that they fell within the ΔpH from 0.8 to 1.1, averaging 0.87 with $\pm$ 0.11 as standard deviation. The rejection limits ($\alpha = 0.05$) were computed as 0.64 and 1.10.

Summary

A routine procedure for the estimation of serum cholinesterase was described. Its principle is placed, like those of the methods having been developed by Michel and Alcalde, on the measurement of pH drop in the buffered substrate mixed with serum. Its technic is simple and efficient, because it employs a comparator to determine pH instead of glass electrode pH-meter. Special caution was taken with regard to the composition of buffered substrate in order that the fall of pH might indicate the approximate amount of acetic acid liberated from acetylcholine by enzymatic hydrolysis. This method has been in use for three years in our laboratory with satisfactory results for the diagnosis of hepatic disturbances. Normal serum cholinesterase activity fell within the ΔpH between 0.8 and 1.1.

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