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STUDIES ON SERUM CHOLINESTERASE*

I. A METHOD FOR THE ESTIMATION OF SERUM CHOLINESTERASE ACTIVITY WHICH IS USEFUL IN THE ROUTINE WORK OF CLINICAL LABORATORY

HIROSHI TAKAHASHI

Department of Clinical Pathology, Yamaguchi Medical School, Ube

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On undertaking our study to evaluate the clinical significance of the variation of the serum cholinesterase activity[1-3] an urgent need for a simple and reliable performance for its estimation was felt, because numbers of serum samples obtained from multifarious patients were to be dealt with within a limited period of time. Several procedures, different in principle, have been put forward for the measurement of this enzume. Ammon's manometric method[4] by means of Warburg's apparatus constituted the standard method for the determination of cholinesterase, but it was too complicated for clinical use. Michel's hydrogen-ionometric method[5] which appeared recently required a glass-electrode pH-meter which was unfortunately liable to obstacles so far as the commercial product available from tht merket of this country was concernnd, although it was simpler in manipulation. Hestrin's method[6] which measured the amount of unsplit acetylcholine colorimetrically after the incubation of the mixture of serum and substrate did not always yield the determination equaling in accuracy to Michel's method, notwithstanding that the procedure was more tedious. A new simple procedure which was based on the principle of Michel's method without the use of electrometric hydrogen-ionometry was therefore devised in our laboratory. The fall of pH after a specified time of incubation was measured in a comparator with phenol red as indicator with respect to the mixture of serum and buffered substrate (acetylcholine) and the blank mixture (water and buffered substrate), and the difference between them (ΔpH) was regarded as representative of the serum cholinesterase activity (Figure 1). In our experience this method was entirely free of the accidents which resulted from troubles with the instrument, and its procedure was so simple that it was especially suited to the mass treatment of a large number of samples within a short period of time. The method has been in use with excellent efficiency in our laboraroy since

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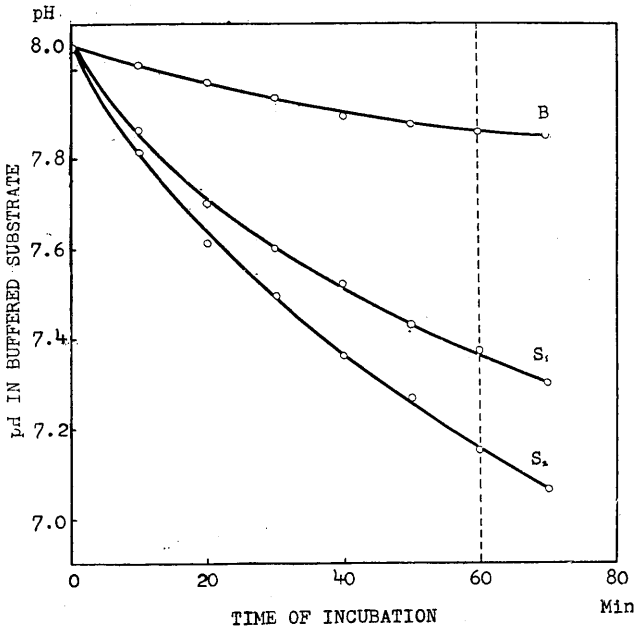

Fig. 1 Fall of pH in the buffered substrate in relation to the time of incubation.
B—Blank tube which refers to the non-enzymatic decomposition of acetylcholine.
S_1 and S_2—Sample tubes, i.e. mixture of serum and buffered substrate. Both enzymatic and non-enzymatic decompositions are responsible for the pH drop in these tubes. Consequently, the difference in the pH fall between the sample tube and the blank tubes represent the activity of cholinesterase in the serun examined.

1951. Lately our method was adapted to a photoelectric colorimeter by Shibata and others[8] independently of Reinhold and his associates[9] who modified Michel's method so that the photoelectric hydrogen-ionometry with phenol red as indicator might be available. Limperos and Ranta[10] also presented a field work procedure based on the same principle with bromthymol blue as indicator.

METHOD[7,11,12]

Reagent

(1) Buffer solution. Introduce 0.6 g. of barbital in about 100ml. of lukewarm distilled water in a 200ml. beaker, warm until complete dissolution, allow to cool to room temperature, add and dissolve 1.9g. of sodium diethyl barbiturate and 2.5g. of sodium β-glycerophosphate. Transfer the solution to a 500 ml

volumetric flask, make to volume with distilled water which has been used for the washing of the beaker, and mix. Check the solution in a comparator with phenol red to indicate that the solution has the pH of 8.3. Add adequate volume of 1N-NaOH or 1N-HCI to adjust the pH to 8.3 when the solution deviates therefrom. Preserve in a stoppered bottle with a few ml. of chloroform. This solution keeps for at least two months in refrigerator.

(2) Acetylcholine solution, 5g./dl. Prepare, immediately before use, an aqueous solution of Ovisot (acetylcholine chloride of Daiichi Kagaku Co., Tokyo, contained in an ampule 0.1g. in amount) in the proportion of one ampule to two ml. of distilled water. This is used as substrate.

(3) Phenol red solution, 40mg./dl. Dissolve 100mg. of phenol red in 3.0 ml. of N/10 NaOH and 7.5ml. of distilled water by gently warming, allow to cool to room temperature, and make to the volume of 250 ml. with distilled water.

(4) Solution of eserine sulfate. i) Stock solution, 1.29×10^{-5}M. Dissolve 0.1 g. of eserine sulfate in 20ml. of distilled water and transfer in to a brown bottle. Keep in a refigerator. ii) Solution for routine use. A small amount of the ten-fold dilution of stock solution is made before use, and preserved in a refrigerator. It is useful unless colored distinctly.

(5) A set of standard series of phenol red tubes for the estimation of pH (pH 8.6–6.4, with 0.2 intervals) and a comparator. Add and mix aliquots of 0,15ml. of phenol red solution (3) to 5,1ml. portions of Sørensen's phosphate buffer solution (pH 8.4, 8.2, 8.0, ……, 6.4) which are contained in the test tubes of the size adequately fit to the comparator. A commercial set is also available and convenient (Kayagaki's (Tokyo) assembly for comparator hydrogenionometry is useful).

Procedure

Into the test tubes B and S which are the same size as those of the standard phnol red series introduce aliquots of 1.5ml. of buffer solution, 3.0ml. of distilled water, 0.15ml, of phenol red solution and 0.5ml. of acetylcholine solution. Add 0.1ml of serum to be determined to tube S, mix by inversion both tubes B and S and incubate at 37°C for an hour. At the end of that time add one drop of eserine solution to each in order to stop the activity of serum chorinesterase.[5] Read the pH b and s of the tubes B and S in a comparator with a standard series of phenol red tubes.* Then the activity of serum cholinesterase is obtained by the equation

$$\Delta pH = b - s.$$

* In case of icteric serum the tubes B and S are required to be compensated for the yellow coloration of bilirubin by placing a tube which contains 0.1 ml of serum diluted with 5.0ml of physiological saline after the standard phenol red tubes.

DISCUSSION[11]

A series of examinations were perfomed with special reference to the following points in order to verify the reliability of our procedure;—(1) the correlation between the fall of pH in buffered substrate and the amount of aceticacid liberated by the decomposition of acetylcholine, (2) the variation of serum cholinestsrase activity with the shift of pH within the range encountered in our procedure, and (3) the accuracy of the pH reading by means of a comparator. The procedure was thought to be greatly dependent on these factors. It was, of course, also compared with the conventional methods for the determination of serum cholinesterase including Michel's and Ammon's procedures. The data obtained will be presented below.

There is a parallel correlationship between the drop of pH *in the buffered substrate and the amount of acetic acid liberated from the acetylcholine by serum cholinesterase.* The buffered solution which is used in this procedure gives the fall of

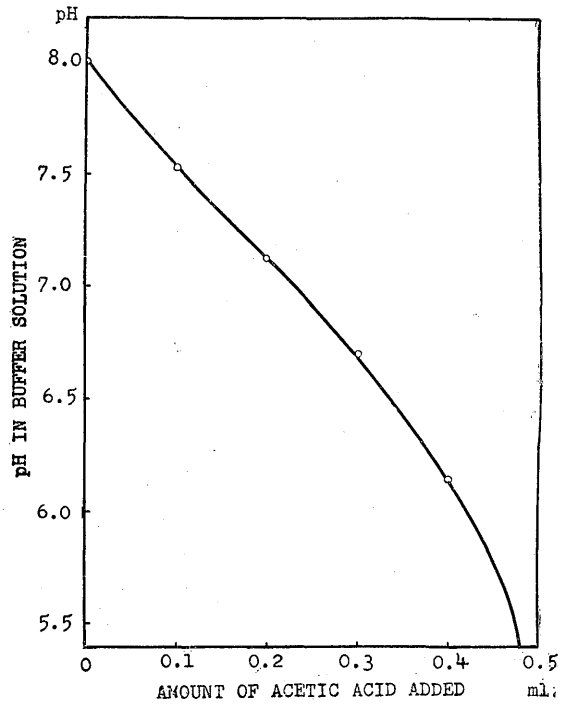

Fig. 2 Shift of pH in the buffer solution after the addition of various amount of N/10 acetic acid.
Into each of the test tubes are introduced 1.5ml. of buffer solution and 3.0ml. of distilled water. The tubes are acidifled by the addition of 0 to 0.5ml. of N/10 acetic acid. pH was measured by a glass-electrode pH-meter.

pH in a linear manner with proportion to the increase in the amount of acid (acetic acid or hydrochloric acid) added to it within the range of pH from 8.0 to 6.5 (Figure 2)*.

The activity of serum cholinesterase is independent of the variation of pH *so far as the shift of* pH *encountered in the sample tube (tube S) is concerned.* This is illustrated in Figure 3, which shows the nearly constant activity of the enzyme activity whithin the limits of pH from 8.8 to 6.7.** The tube S gives

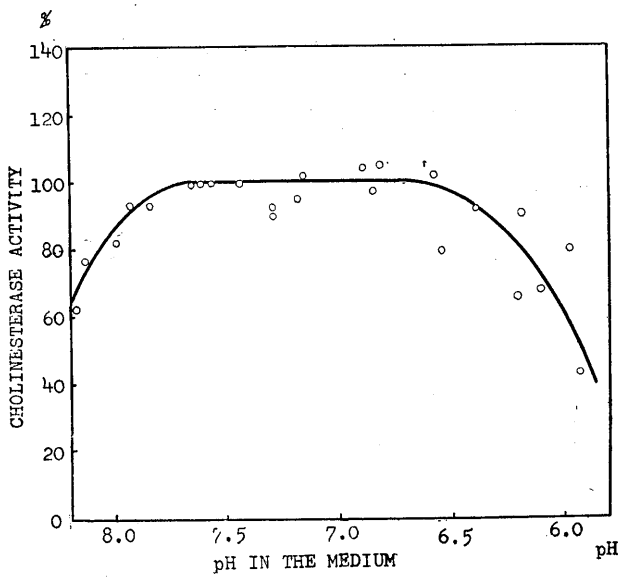

Fig. 3 The activity of serum cholinteserase in relation to the variation of pH in the medium.

A series of buffer solutions varying in pH from 5.0 to 9.0 were prepared in the same way as in the experiment depicted in Fig. 2, and cholinesterase was determiaed at various pHs by a glasselectrode pH-meter with human blood serum 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 flnal 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 one-hour's incubation at 37°C.

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* A suggestion for the composition of the buffer solution was obatined from Alcalde.[13] The buffer capacity of the solution is superior to the barbital buffer solution, particularly in the range of pH below 7, because of the glycerophosphate which maintains the alkalinity of the solution, thus preventing its abrupt fall of pH when acid is added.

** The pH of the buffered substrate used in the current methods for the determination of serum cholnesterase varies 8.5[13], 8.0[10], 7.6[15], 7.0[16] and 6.4[17] according to the different authors. This will be an evidence for the wide range of optimum pH for serum cholinesterase activity although the difference in the principle of the procedure has to be taken into consideration.

pH 8.0 shortly after its preparation (addition of serum to buffered substrate), and it does not descend as low as pH 6.8 even in the case of the serum of high cholinesterase activity (healthy peson). Its pH falls accordingly within the range which results in little alteration in the enzyme activity throughout the period of incubation. This fact in conjunction with the presence of parallel correlationship between the dop of pH in the buffered substrate and the amount of acetic acid liberated, as mentioned above, assures the validity of ΔpH as a index of the activity of serum cholinesterase, because ΔpH is expected to mirror correctly the amount of acetic acid produced by the action of the enzyme. By the same token a linear correlation between the fall of pH and the amount of serum cholinesterase (ΔpH 0–1.3) is depicted in Figure 4.

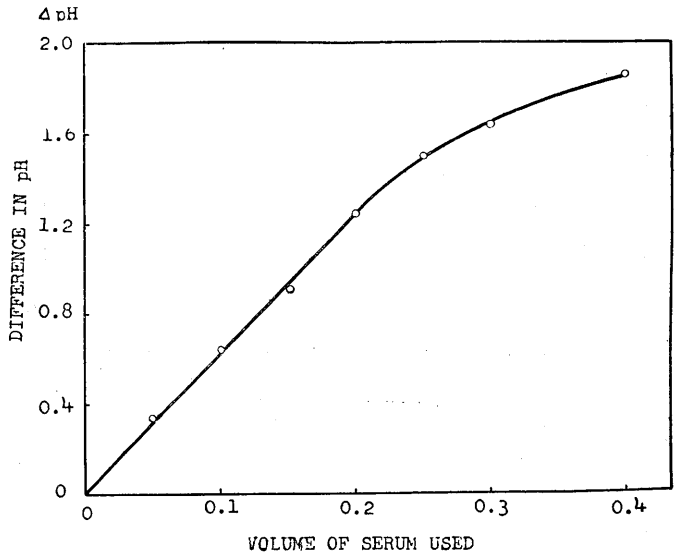

Fig. 4 Relation between ΔpH and the amount of serum used for the estimation of serum cholinesterase.
Amount varying from 0 to 0.4ml. of serum were substituted for the 0.1ml. of serum which was specified in our procedure. A glass-electrode pH-meter was used for the measurement of pH.

The pH *reading by means of a comparator is reliable.* As shown in Figure 5 the ΔpH (C), the ΔpH when pH of the sample and blank tubes was read in a comparator, compares well with the ΔpH (G), the ΔpH when pH was read by a glass-electrode pH-meter. There is a linear correlationship between them, although ΔpH (C) is larger as much as ten per cent than ΔpH (G). Slight deviation of pH reading in a comparator to the higher side and similarly minute deviation of the comparator reading to the lower side in the pH range above

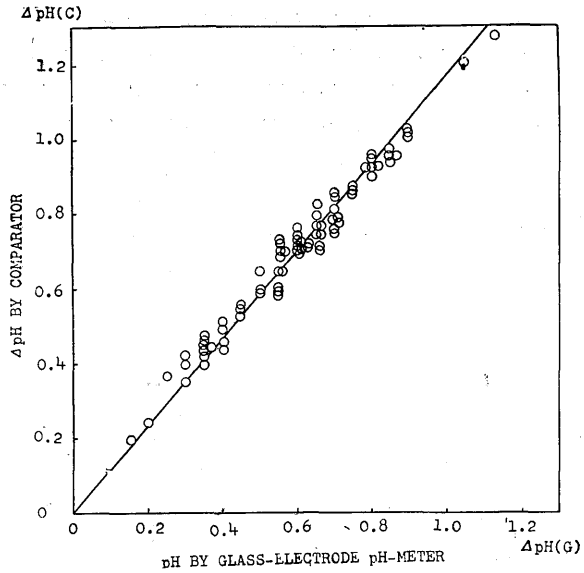

Fig. 5 The correlationship of ΔpH(C) obtained by comparator to ΔpH(G) measure by a glass-electrode pH-meter in our procedure.
Both ΔpHs were determined simultaneously with the same mixtures of serum and buffered substrate.

and below 7.1, respectively, as compared with the pH reading by means of a glass-electrode pH-meter are supposed to be responsible for the ten percent discrepancy. ΔpH (C) divided by 1.1 falls into the limits ΔpH (G) $\pm$0.05. The comparator reading of pH adopted in our procedure is not thought to invalidate the accuracy and reliability of estimation, although at first sight it may be feared to impair them seriously.

The concentration of acetylcholine in the buffered substrate immidiately before it is incubated is 0,026 Mol. Higher concentration entailed little augmentation in ΔpH*. The blank tube B is necessary for the elimination of non-enzymatic hydrolysis of acetylcholine which is appreciable above pH 7.1. A thermostat of 37°C is also required, because the enzyme activity is comparaitvely sensitive for the fluctuation of temparature (A rise or fall of 1°C entails the 2 per cent increase or decrease in the activity)**. A sufficient amount (10^{-6}M)

* The concentration of 0.015M–0.200M of acetylcholine is commonly used.[4)5)13)] Ammon[4)] and Michel[5)] employ 0.015M, and De La Huerga[18)] 0.03M, while Tamai[15)] regards the concentration over 0.05M as optimum (when 0.025M is used the activity is lowered by 5 per cent).

** ΔpH increases with the rise in temperature of incubation (for an hour) within the range from 13 to 46°C. The enzyme is inactivated by the incubation at 40°C for five minutes and rapidly loses its activity at 55°C. The temperature of 37°C is thought to be adequate. Michel employs the incubation at 25°C in order to preclude the drop of pH of the buffered substrate by the non-enzymatic decomposition of acetylcholine because his buffer solution is not sufficiently strong.

of eserine solution ought to be used to stop the enzymatic decomposision of acetylcholine which takes place within the period between the withdrawal of the tubes from the thermostat and their pH reading. The addition of calcium, magnesium and potassium (as activator) did not result in the significant rise in ΔpH.

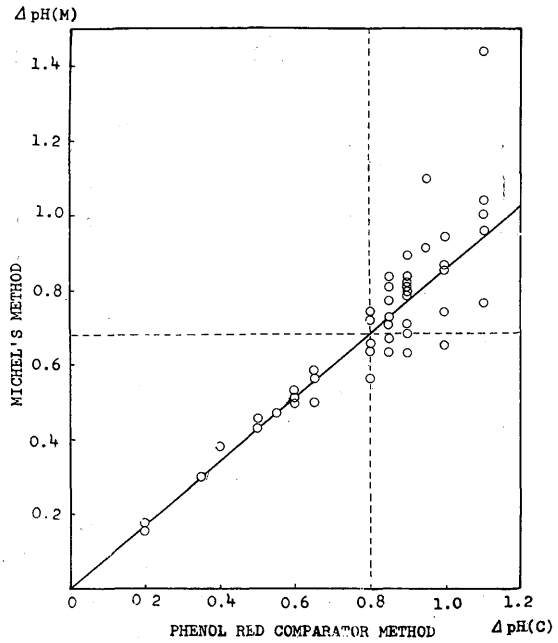

Fig. 6 Comparison of our procedure (ΔpH(C)) with Michel's method (ΔpH (M)).
The same serum was subjected to both the phenol red comparator method and Michel's method.

The ΔpH of our procedure exhibits a satisfactory agreement with the determinations of the pre-existent authorized methods for the estimation of serum cholinesterase. Figure 5 reveals the result of the comparison with Michel's method. There is an approximately linear correlation between our procedure (ΔpH (C) and Michel's (ΔpH (M)) (coefficient of correlation: +0.95), and in the range of ΔpH (C) below 0.8 the following linear equation is obtained, because conformation is parrticularly excellent (coefficient of correlation: +0.98) therein.

$$\Delta\text{pH}(\text{C}) = \frac{5}{4} \times \Delta\text{pH}\ (\text{M})$$

The ΔpH (C) varies similarly in good agreement with the determination of Ammon's manometric method (μLCO_2) as clearly illustrated in Figure 6 (coef-

cient of correlation: +0.90)*. The parallelism is almost complete in the range of ΔpH(C) less than 0.8 (coefficient of correlation: +0.95), giving approximately a linear equation:

$$\Delta\text{pH(C)} = 150/0.8\mu\text{LCO}_2$$

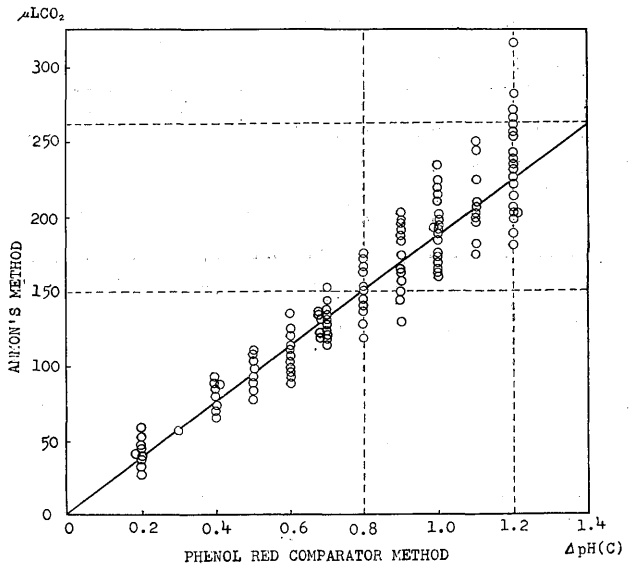

Fig. 7 Comparison of our procedure (ΔpH(C)) with Ammon's manometric method (μLCO_2).
The same serum was subjected to both the phenol red comparator method and Ammon's method.

In our procedure the serum cholinesterase activity of a normal healthy person falls within the range of ΔpH 0.8–1.1. Observation on healthy males and famales, sixty in total number, throughout a year disclosed 0.87 of ΔpH on an average with 0.11 of standard deviation and 0.64–1.20 of rejection limits (α=0.05). Variation due to the difference in age and sex was not appreciable, but a slight degree (ΔpH: 0.05–0.10) of seasonal fluctuation (higher in winter than in summer) seemed to be noted.

Summary and Conclusion

A simple procedure for the estimation of serum cholinesterase which is

* The author is very much grateful to Prof. Kiyoshi Hiraki and his associates who kindly conducted the comparison of our procedure with Ammon's method.

applicable to the routine work of clinical biochemistry was presented. It was based on the same principle as that of Michel's method. The drop of pH in the mixture of serum and buffered substrate (acetylcholine chloride in barbital glycerophosphate buffer) after one hour's incubation at 37°C was measured in a comparator with phenol red as indicator instead of a glass-electrode pH-meter. The composition of the buffered substrate was so adjusted that its pH might fall exactly in proportion to the amount of acetic acid released from acetylcholine by enzymatic (cholinestrase) action within the range which did not entail the significant alteration in the enzymatic activity. The procedure was in good parallelism with Ammon's and Michel's. Four year's experience with this procedure in our laboratory revealed that it was satisfactorily efficient. Normal range of serum cholioesterase activity in this procedure is ΔpH from 0.8 to 1.1.

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