A New Colorimetrical Determination of Cholinesterase*

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Recently, S. Hesterin[1] published a new chemical method for the colorimetrical determination of acetylcholine (Ach) using hydroxylamine as a reagent.

In view of the physiological importance of Ach we have made additional experiments and conducted a reinvestigation, verifying Hesterin's method as acceptable.

Furthermore based on the above, we have perfected a new method for determination of cholinesterase.

(I) Colorimetrical determination of acetylcholine

Hesterin's procedure is as follows;

1) 2 cc of alkaline hydroxylamine reagent (1 cc 2 M hydroxylamine hydrochloride solution and 1 cc 3.5 M sodium hydroxide solution) was added to 1 cc of standard Ach solution, and the mixture was kept for one minute or more before the next step.

2) The solution was then acidified with 1 cc of hydrochloric acid (1 vol. concentrated hydrochloric acid S. W. 1. 18+2 vol. of water).

3) Finally 1 cc of 0.37 M ferric chloride in 0.1 N hydrochloric acid was added.

4) The density of the purple-brown color was promptly determined at 540 mμ.

Experimental Results

1) Relationship between the concentration of Ach and extinction.

It is evident that concentration and extinction are proportional, Lambert-Beer's law is applicable (Fig. 1) $K=2.570$ (by least square method). By spectrum analysis, we observed the greatest absorption at 530 mμ in the color of final solution. (Fig. 2)

It is consistent with Hesterin's report.

2) Stability of the color;

The color of final solution fades in accordance with time; The density of the color diminishes, as the temperature rises.

In our case, (with the density of color immediately after the procedure placed at 100) the color density of the solution stored in the refrigerator stood at 98 showing but a negligible decrease, whereas the solution kept at room temper-

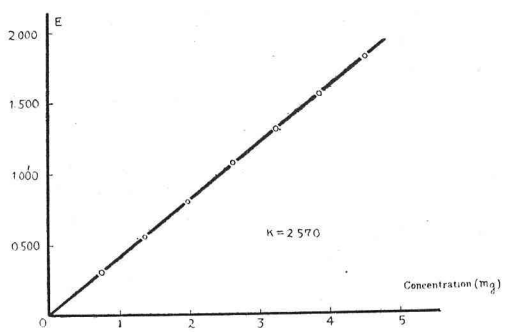

Fig. 1
Relationship between the concentration of Ach and extinction

* Originally published in Japanese (Sapporo Ikadaigaku Kiyo **2**, 7 (1951).

1) Hesterin, S.: J. Biol. Chem., **180**, 249 (1949).

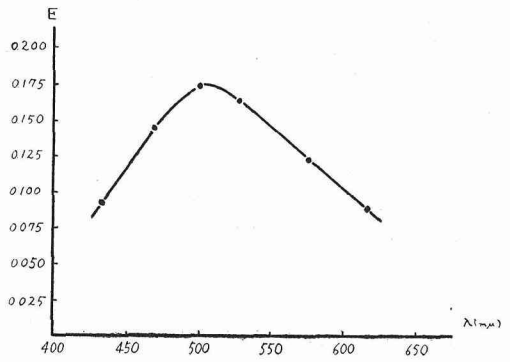

Fig. 2
Extinction curve

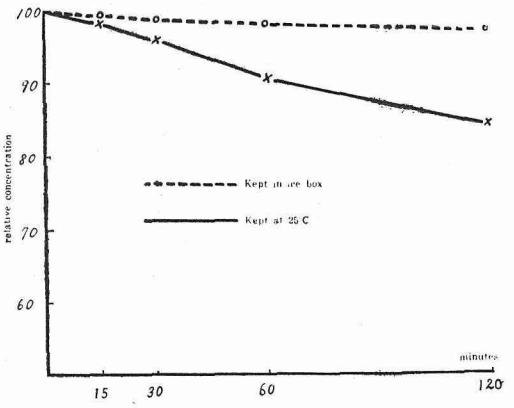

Fig. 3
The influence of temperature and time for fading

ature (25°C) receded to 90. (Fig. 3)

The colorimetry, therefore, must be handled as rapidly as possible. It would also be to an advantage if the solutions be kept in ice water.

3) Reagents.

We conducted a reinvestigation of various reagents reported by Hesterin preparatory to our experiments. In regards to the said reagents, the quantitative relations are entirely acceptable and the results are highly satisfactory.

However, we believe that there are a few points in the procedure that require improvement:

i) Stability of alkaline hydroxylamine

Though Hesterin prescribed alkaline hydroxylamine kept at room temperature (25°C) for three hours, in our experiments, the color density of the above was slightly lower than that of immediately after the reaction. Therefore it is our belief that the said solution should be prepared, not too long before an experiment is to be conducted.

ii) According to Hesterin's procedure and in the later steps of the reactions, the solution is acidified with hydrochloric acid and then ferric chloride is added, to produce color. Instead of the prescribed hydrochloric acid we used sulfuric acid (1 vol. concentrated sulfuric acid S. W. 1.98+9.5 vol. of water). We achieved the following favorable results.

a) In the bland contrast, the final color is a very light yellow and when compared with results obtained using hydrochloric acid it is many shades lighter.

b) Sensitivity of reaction between hydrochloric acid and sulfuric acid is unchanged when corresponding contrasts are used.

As mentioned above the use of sulfuric acid in place of hydrochloric acid has its advantages; namely it diminishes error based on the difference of color in clinical research.

4) Sensitivity and error.

The upper limit of measurement exceeds 1 mg and the lower limit is about 6 γ per cc of the final solution with Pulfrich's photometer.

This is generally consistent with Hesterin's results (the upper limit 5 μM, the lower limit 0.04 μM per cc of the final solution).

However, the physiological amounts of Ach are far too low to be measured by this method.

Measurement error is within 3%.

(II) The determination of cholinesterase

In the past, the measurements of activity of cholinesterase (ChE) have been variously described by Minz[2] Stedman[3] Hawes[4] etc.

Their methods respectively have advantages and defects in their sensitivities and errors. Thus at present, Ammon's method[5] has been prevalent.

However, as the later requires a special apparatus, it could hardly be said that it is universal.

Therefore we applied the colorimetrical determination of Ach reported by Hesterin to ChE-determination.

Namely, when a certain concentration of Ach is added to the enzyme solution, which is extracted with phosphate buffer, part of it is resolved by ChE into choline and acetic acid. As a result, by colorimetrical determination of Ach remaining in the solution, it enables us to ascertain the resolved amount of Ach.

We have conducted an extensive experiment concerning the determination methods and factors.

Experimental Results

(A) Various factors affecting the color relation of Ach.

It is absolutely necessary that we determine whether, there is an interference by salts, contained in organs and tissues, and by protein praecipitants, prior to the determination of ChE-activity in preparation of the use of this method.

The following experiment was conducted from this point of view.

1) Influence of salts.

We determined the influence of salts to the Ach color reactions by testing 1 cc each of a 2% solution of the following KCl, NaCl, $CaCl_2$, $NaHCO_3$, $MgSO_4$, KH_2PO_4, Na_2HPO_4. As a contrast 1 cc of distilled water was used inplace of salts. (Fig. 4)

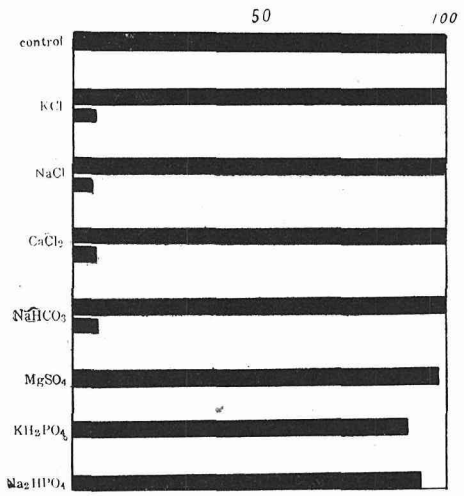

Fig. 4
The influence of salts

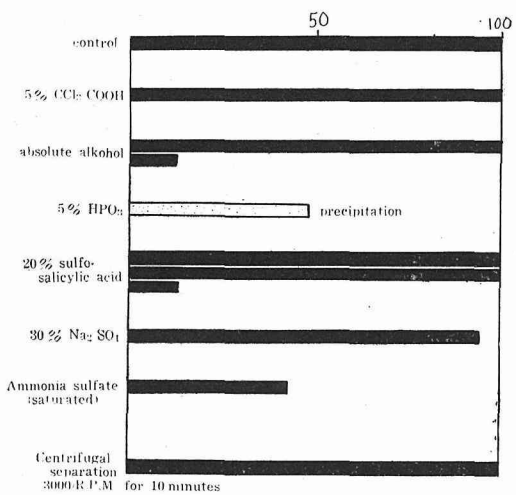

Fig. 5
The influence of protein-precipitants

2) Minz, B.: Arch. exp. Path. Pharmak., **168**, 292 (1932).
3) Stedmann, E.: Biochem. J., **25**, 1147 (1931).
4) Hawes, R. C.: J. Lab. Clin. Med., **26**, 845 (1941).
5) Ammon, R.: Pflügers Archiv, **233**, 486 (1934).

As shown in Fig. 4, except for KH_2PO_4 and Na_2HPO_4, results of others are slightly above the contrast. However, in spite of the fact that very little influence may be observed by the remainder, potassium biphosphate and sodium phosphate bibasic, obviously have a slight inhibiting effect. Though this phenomenon has been described by Hesterin etc., an inhibition of this degree will not interfere with its use as a buffer solution to determine ChE.

The fact that these ions have hardly a perceptive effect on the present reaction, may prove significant in a research, in regards to the influence of various ions on enzyme activity.

2) Problems concerning the praecipitation of protein.

i) Protein-precipitants.

We made a research, in accordance with the procedure described in the previous paragraph, utilizing main protein-precipitants. (Fig. 5)

As a result, and as described by Hesterin, trichlor-acetic acid alone can be used effectively as a protein-precipitant.

ii) As it is possible to completely remove protein dissolved in this solution, by acidifying the solution in its later stage of reaction, thereby causing it to precipitate and henceforth remove it by the simple process of centrifugal separation; the use of a protein-precipitant is not required. Moreover a protein test with sulfosalicylic acid added to the supernatant showed negative results.

iii) We then commenced to test whether a loss of color components had occurred that may have been caused by centrifugal separation.

A: Ach 1 cc+vagostigmin 1 cc+enzyme solution 1 cc+phosphate buffer 1 cc

B: Ach 1 cc+vagostigmin 1 cc+Aq. dest. 1 cc+phosphate buffer 1 cc

C: enzyme solution 1 cc+Aq. dest. 2 cc+phosphate buffer 1 cc

Table 1

	Extinction (E)			Mean
A	0.252	0.252	0.250	0.251
B	0.220	0.215	0.218	0.218
C	0.033	0 033	0.033	0.033

Each group was kept at O °Cfor 30 minutes in ice water to holding down ChE activity completely. Results of measurement of E, $A \fallingdotseq B + C$.

It is apparent that no loss what-so-ever of E was sustained, by centrifugal separation of protein in the last stages of the reaction. Thus proving the applicability of this method in measuring ChE.

3) Coloration of enzyme fluid.

The majority of enzyme extracts in themselves hardly show any color. However as an example serum, mussel shell muscles etc. show a very slight color. C) of the above paragraph indicates same.

At present the cause is undetermined. However in view of the fact that the quantities of Ach in organs are generally known to be extremely slight[6),7)] it would be difficult to attribute the above to Ach. Wether it is caused by reactional positive components other than Ach or whether it is because of the pigment in the organ itself is unknown. However in a case like this, by providing a contrast as described above, it is highly possible to adapt the present reaction to the determination of ChE.

(B) Determination of cholinestrase.

1) Buffer:

6) Feldberg, W. & Schild: J. Physiol. **81**, 37 (1934).

7) Nochimouski, C.: J. Physiol. Path. gén., **35**, 746 (1937).

We found Ringer-natrium bicarbonate buffer can be used to buffer acetic acid formed as a result of ChE splitting Ach, and further discovered that phosphate as a buffer inhibits the present reaction slightly. But due to the fact that it has a wider pH range and is very effective as a buffer, phosphate buffer's virtues supersedes its defects. In our experiments, we combined 1 cc of enzyme solution, 1 cc of Ach solution with 2 cc of phosphate buffer, which held at 38°C for 30 minutes in abath, did not show any change in pH.

We must say that the merits of phosphate buffer, in comparison to Ringer-natrium bicarbonate, exceed particularly in the determination of influence of pH in regards to enzyme-activities.

In the past, due to its origin, it was only possible to use phosphate buffer in bioassay. It was impossible to utilize others. Thus it was considered unpractical to conduct a comparing research, over a wide pH field, in regards to the value of ChE. Moreover there was no way to make a conclusive research. Hereafter making use of the present method, it is highly satisfactory to be able to enlighten this unexplored field.

2) Concentration of substrat;

The concentration of Ach in the determination of ChE, varies in accordance with investigators[8) 9), 10), 11)]. We have adhered to Tamai[12)]. According to his reports, as a result of an extensive research concerning concentration of substrat in ChE measurements of serum and erythrocyten at pH. 7.4, he concludes that 0.025 M Ach in Serum (pseudo ChE) and 0.0025 M Ach in erythrocyten (true ChE) are the best.

In our laboratory Konno[13)], adapted the said established connections to other organs and discovered that a parallel exists between the above concentration of substrat and previously reported specialities of various organs. Therefore on their heels, we used respectively 1 cc of 0.0025 M Ach for pseudo-ChE and 1 cc of 0.025 M Ach for true-ChE to determine the measurements.

In addition, we adopted the following method so as to convenience colorimetry.

i) Tissue containing pseudo-ChE.

Enzyme solution 1 cc+0.025 M Ach 1 cc+phosphate buffer 2 cc at 38°C for 30 minutes.

Take 1 cc from above, add 1 cc distilled water. Determine Ach color density.

ii) Tissue containing true-ChE.

Enzyme solution 1 cc+0.0025 M Ach 1 cc+phosphate buffer 2 cc at 38°C for 30 minutes.

Take 2 cc of above. Determine Ach color density.

In determination of ii), the final color density is so low that it necessitates the use of twice the amount of solution to be tested to gain favorable results, and the above is merely to avoid and to eliminate any possibilities of a high degree split due to an over existence of ChE.

3) Natural splitting of Ach.

It must be noted that a natural splitting of Ach may ensue when ChE is brought into contact with the solution at 38°C for 30 minutes in a bath.

The above has been noted and reported by many experts. We have noted that 0.025 M Ach (at 38°C for 30 minutes) decomposes naturally at the rate of 6.3%.

We took Ach solution as our sole objectiv to determine and corrected the results on the determination of ChE.

4) Preparation of Enzyme solution:

The conditions under which enzyme solution is to be prepared must be highly constant our method is as follows.

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8) Alles, G. A. & Hawes: J. Biol. Chem., **133**, 375 (1940).

9) Easson & Stedmann, E: Proc. Roy, Soc., London. Ser. B, **121**, 142 (1934).

10) Glick. D: Biochem. J.,**31**, 521 (1937).

11) Mendell, B. & Rudney, H.: Biochem. J., **37**, 473 (1943); Science, **100**, 499 (1944).

12) Tamai, A.: J. Jap. Biochem. Soc., **22**, 32 (1950).

13) Konno, A.: Unpublished.

Add pH 7.4 phosphate buffer to 4 g of tissue, and grind to a brei. Entire amount 25 cc prepare, so as 1 cc of extract will contain 0.16 g of tissue. The Enzyme extract solution is kept in cold storage for 24 hours allowing for exudation.

Separate by centrifugation at 3,000 revolutions for 30 minutes. Use 1 cc for test.

The above preparation is the highest concentration to be attained through various tissues.

Among certain varieties of tissue, especially in those with a high ChE value it is necessary to select a solution with the exact concentration suited for the present determination method.

(C) Method of determination of ChE.

Based on the above mentioned experiments, we consider the following method adequate for the determination of ChE.

1 cc Enzyme solution (0.16 g/cc) is added to 1 cc of 0.025 M or 0.0025 M Ach solution. Add 2 cc of phosphate buffer, hold at 38°C for 30 minutes in the bath. Take 1 or 2 cc from above and add 2 cc alkali hydroxylamine, then follow Hesterin's method. Take final color solution and separate by centrifugation at 3,000 R. P. M. for 10 minutes and run a colorimetry of supernatant. As a contrast use 2 cc of distilled water in Ach reagents.

1) 1 cc of enzyme extract (0.16g/cc)+1 cc of Ach+2 cc of phosphate buffer

↓

38°C, 30 minutes.

take 1 cc (in the case of pseude-ChE) or 2 cc (in the case of true-ChE)

↓

Color reaction of acetylcholine

↓

zentrifugate 3000 R. P. M. for minutes

↓

colorimetry ····· (E)

2) with Ach as sole objective;

1 cc of Aq. dest.+1 cc of Ach+2 cc of phosphate buffer

↓

38°C, 30 minutes

take 1 or 2 cc

↓

color reaction

↓

colorimetry ····· (E_1)

amount of split acetylcholine $= 2.570 \times (E_1 - E)$ mg

Concerning tissues in which, as mentioned previously, the enzyme solution itself shows a color reaction, evaluation of enzyme extract itself should be added and corrected.

(D) Evaluation of ChE in animal tissues.

In order to test our new method of ChE estimation by actually applying same, we ran an estimation on ChE of liver of dogs, liver, cerebrum, and kidney of rabbits and obtained the following as plotted in Table 2.

Table 2

	liver	cerebrum	kidney
dog	7.337	—	—
rabbit	0.988	1.323	0.269

Figures on table 2 indicate Ach mg/38°C, 30 minutes, split by ChE contained in 0.16 g of tissue. Which shows that dog tissue has an exceptionally higher value than rabbit.

And in rabbit the activity rating, showed in following order, cerebrum>liver>kidney.

Wakabayashi, Sato[14] using Ammon's method have observed that dog's liver has an extremely superior activity.

Also they have reported the order of activity in rabbit follows cerebrum>liver>kidney, order, which coincides with our results.

Contrary to the need of special apparatus required in Ammon's method, such as vessels and manometers which are essential in the determination of ChE by this method, the present method is easier as it is not encumbered by any special apparatus. Thus making it ideal for clinical research.

Moreover in regards to sensitivity, in the Ammon's method the limit is from 0.5 mg to 0.04 mg. The lower limit of estimation being the same as in the present method while as the upper limit comes only half ways. With a wider range of estimation we believe the present method has an advantage to the Ammon's method.

As far as errors are concerned, though we are not able to state definitely, there seems to be no great difference.

However even in the present method, the fact remains that there are two defects, namely fading of color and non speciality in Ach.

Actually, in animal experiments, this non speciality in Ach can be corrected and minimized by setting a contrast as described in previous paragraphs, so that it does not present a serious problem. Also by making conditions as constant as possible and by speeding up of determinations, the loss of color can easily be avoided.

Above all the procedune is simple and the fact that special apparatus is not required makes it superior to Ammon's method as a clinical research method.

In conclusion we wish to add that with the same principle it is further possible to determine the activity of cholinacetylase. Efforts are being made in this direction.

Summary

1. We conducted additional research of Hesterin's method and made improvements on reagents.
2. Utilizing Hesterin's method we were able to verify various factors in biological determinations and have arrived at a conclusion that the method can be brought to use.
3. We established a method of quantitative determination on ChE and determined ChE value of two or three animal tissues.
4. The new method on ChE determinanion shows results parallel to Ammon's method.

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14) Wakabayashi, G. & Sato, M.: J. Jap. Biochem. Soc., 21. 2, 81 (1949).