Modification of Michael's Method for Determination of Serum Acetyl Choline Esterase Activity

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The activity of acetylcholine esterase (CHE) is a well-known indicator for organo-phosphorous insecticide poisoning and liver function test in the clinical biochemistry test of men and experimental animals.

The methods for determinating CHE activity are grossly divided into 1) CO_2 detecting method (AMMON, 1934), 2) ΔpH method (MICHAEL, 1949; SHIBATA & TAKAHASHI, 1953; ECOBICHON, 1970), 3) choline titration method (HUERG, 1952), 4) thiocholine method (MIYAMOTO, et al., 1972).

CO_2 detecting method gives highly precise values, but it is not convenient because it needs large apparatus.

Thiocholine method has excellent characterestics that it gives highly precise values in a short time, but the substrate is so expensive and substrate specificity is so clear that it may be used only for analytical purpose.

As ΔpH method, 3 different types have been used: 1) direct measurement of pH with glass electrode (MICHAEL, 1949) 2) colorimetry with indicator (SHIBATA & TAKAHASHI, 1953) 3) alkaline titration in optimum pH of CHE with pH-stat (ECOBICHON, 1970). These ΔpH methods commonly give consistent values in convenient process, and are popular for clinical screening purposes, for which total CHE activities are needed without clearing substrate specificity and origins of the enzyme, because of its cheapness, conveniency, precission and wide applications.

But there are some troubles in comparing these values from ΔpH method between species and methods. For, the data have some extent of wide variations due to the buffer action and temparature of incubation medium and variations of pH of the sample.

So, improvement of this method might be important in order to gain the precise, stable and comparable values which are possible to be compared between species and methods.

These improvements shown in this report are to be calculated new corrections based on CHE response in the incubation medium and to be gained constantly stable, precise and comparable values. Rat serum CHE determined by this method are also shown in this paper.

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MATERIALS AND METHOD

Reagent:

1) Buffer: Aquous solution containing sodium barbital 0.006M, KH_2PO_4 0.001M, NaCl 0.030M. adjusted accurately to pH 8.0 by titration with 0.1N HCl. The solution is diluted with an equal volume of distilled water before use.
2) Substrate: 0.165M acecylcholine chloride in distilled water.

Method and procedure:

A tenth mililiter serum is put in 2 ml buffer solution. Pre-incubation 10 min. at 37°C, and the initial pH (pH_i) is measured with pH meter (Toshiba-Beckmann expandmatic pH glass electrode). Then 0.2 ml. substrate solution is added with mixing and incubation is reset. After 1 hour incubation, the final pH (pH_f) is determined.

Cholinesterase activity (ΔpH/hr.) is calculated by

$$\Delta pH/hr. = (pH_i - pH_f - b) \times i$$

where b is correction factor for non-enzymatic hydrolysis corresponding to pH_i and pH_f, and i is correction factor for variations in ΔpH/hr. with pH, corresponding to pH_i.

These corrections are determined by the examinations as to linearity between incubation time and ΔpH without correction, non-enzymatic and enzymatic hydrolysis of acetylcholine under different pH conditions.

Standard curve for trans-value to the unit of μ mole/ml/min. or hr. is gained by the titration of incubation medium with acetic acid.

RESULT AND DISCUSSION

Relation between ΔpH and time of incubation seems almost linear till 180 minutes (Fig.1).

Non-enzymatic hydrolysis of acetylcholine for an Hour is shown in Fig. 2. The function between non-enzymatic hydrolysis of acetylcholine (y: ΔpH) and initial pH of the incubation medium (x) approximates to the hyperbola curve of

$$y = \frac{0.099}{8.814 - x} - 0.020$$

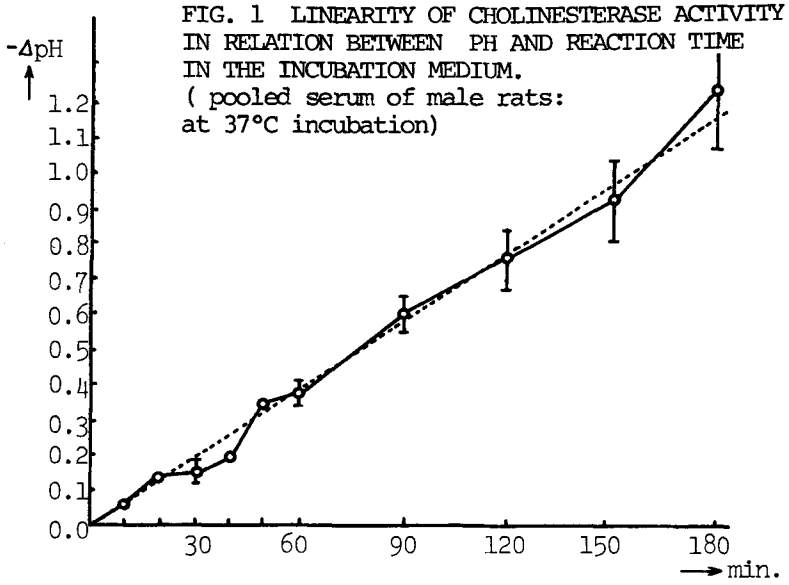


FIG. 1 LINEARITY OF CHOLINESTERASE ACTIVITY IN RELATION BETWEEN PH AND REACTION TIME IN THE INCUBATION MEDIUM.
(pooled serum of male rats: at 37°C incubation)

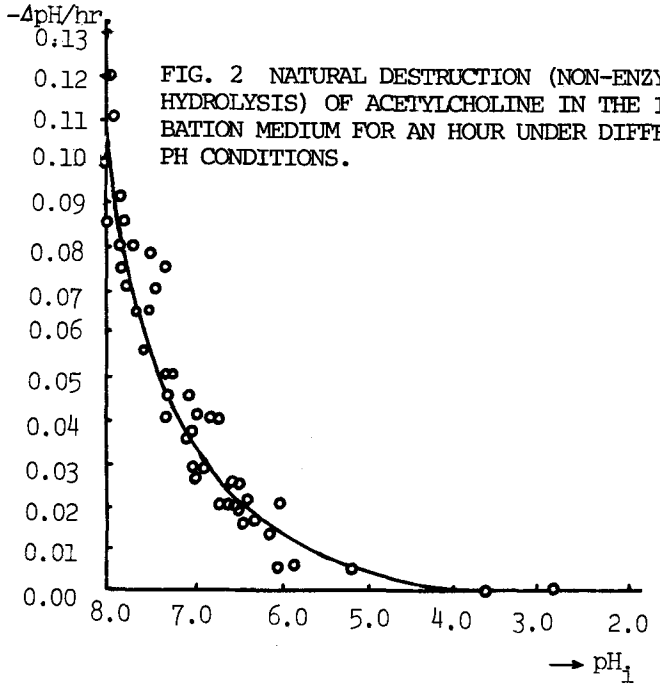


FIG. 2 NATURAL DESTRUCTION (NON-ENZYMATIC HYDROLYSIS) OF ACETYLCHOLINE IN THE INCUBATION MEDIUM FOR AN HOUR UNDER DIFFERENT PH CONDITIONS.

Non-enzymatic hydrolysis of acetylcholine under the state with enzyme is corrected by b correction which is led by the following formula:

$$b = \frac{y(pH_i)}{pH_i - pH_f} \int_{pH_i}^{pH_f} y\,dx = \frac{y(pH_i)}{(pH_i - pH_f)^2} \left[0.099(\log|x-8.814|) - 0.020x\right]_{pH_i}^{pH_f}$$

This formula is based on a hypothesis that the speed of non-enzymatic hydrolysis might be a primary function to the time. In the process of solving this formula, time factor disappears into the factors pH_i and pH_f.

TAB. 1 B CORRECTION CALCULATED BY THE FORMULA ABOVE STATED.

pH_i / pH_f	8.0	7.9	7.8	7.7	7.6	7.5	7.4	7.3	7.2	7.1
7.9	0.109									
7.8	0.034	0.057								
7.7	0.022	0.028	0.047							
7.6	0.016	0.018	0.023	0.039						
7.5	0.012	0.013	0.015	0.019	0.033					
7.4	0.010	0.010	0.011	0.012	0.016	0.028				
7.3	0.008	0.008	0.008	0.009	0.011	0.014	0.025			
7.2	0.007	0.007	0.007	0.007	0.008	0.009	0.012	0.022		
7.1	0.006	0.006	0.006	0.006	0.006	0.007	0.008	0.009	0.019	
7.0	0.006	0.005	0.005	0.005	0.005	0.005	0.006	0.007	0.009	0.017
6.9	0.005	0.005	0.004	0.004	0.004	0.004	0.005	0.005	0.006	0.008
6.8	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005
6.7	0.004	0.004	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004
6.6	0.004	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
6.5	0.003	0.003	0.003	0.003	0.003	0.002	0.002	0.002	0.002	0.003
6.4	0.003	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002
6.3	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
6.2	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
6.1	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
6.0	0.002	0.002	0.002	0.002	0.002	0.002	0.001	0.001	0.001	0.001
5.9	0.002	0.002	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001
5.8	0.002	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001
5.7	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.6	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.5	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.4	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.3	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.2	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.1	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5.0	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

B corrections calculated are shown in Tab. 1. Enzymatic hydrolysis of acetylcholine with pH of incubation medium corresponding to pH_i is shown in Fig. 3. The curve means relative enzyme acti-

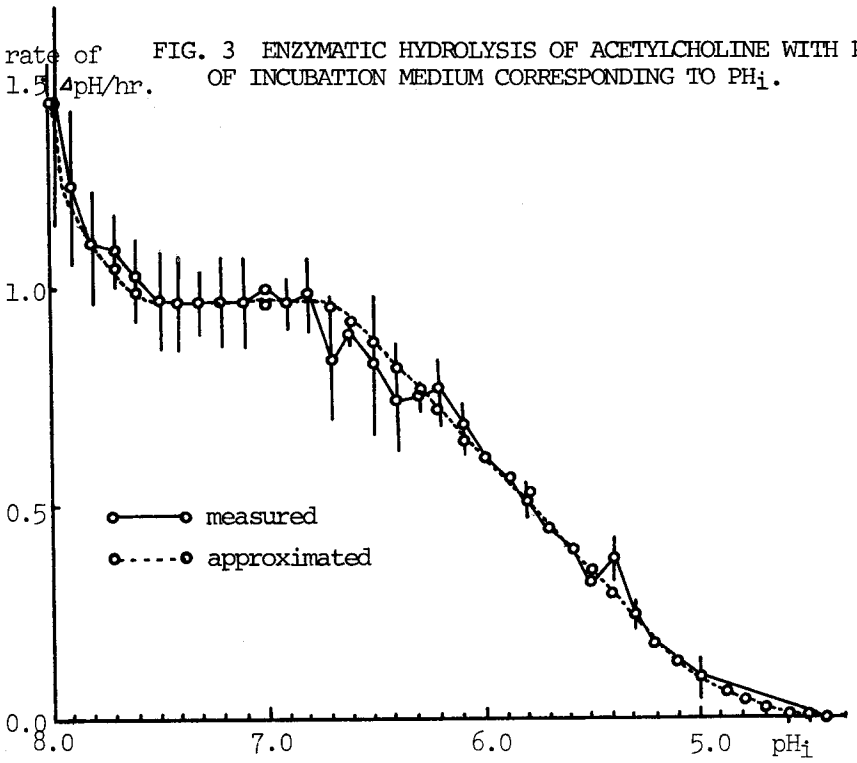


FIG. 3 ENZYMATIC HYDROLYSIS OF ACETYLCHOLINE WITH PH OF INCUBATION MEDIUM CORRESPONDING TO pH_i.

vity as shown in Tab. 2 to the values under 7.00 of pH_i which is common in each 6 experiment that contained 40 to 88 measure points to gain these values. Correction i means the factor of transforming the values of enzymatic hydrolysis under 8.00 of pH_i, and is calculated by the following formula:

$$i = \Delta pH\ (pH_i=8.00) / \Delta pH\ (pH_i=x)$$

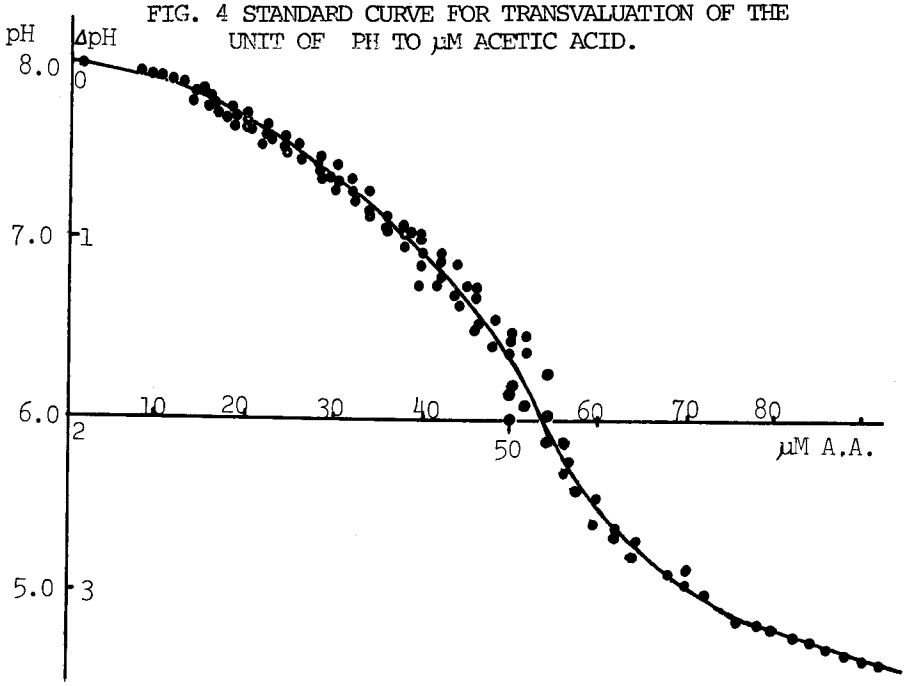
TAB. 2 RELATIVE ENZYME ACTIVITY AND I CORRECTIONS

pH_i	i correction 1)	rate of ΔpH/hr. 2)	pH_i	i correction 1)	rate of ΔpH/hr. 2)
8.0	1.00	1.43±0.30	7.2	1.46	0.98±0.11
7.9	1.15	1.24±0.19	7.1	1.47	0.97±0.11
7.8	1.29	1.11±0.13	7.0	1.43	1.00±0.00
7.7	1.30	1.10±0.09	6.9	1.47	0.97±0.06
7.6	1.39	1.03±0.10	6.8	1.44	0.99±0.09
7.5	1.46	0.98±0.13	6.7	1.72	0.83±0.14
7.4	1.47	0.97±0.12	6.6	1.59	0.90±0.03
7.3	1.46	0.98±0.09	6.5	1.72	0.83±0.18

1) $(pH_i - pH_f - b)^{-1}$, 2) mean±S.D.

Standard curve made by acetic acid titration in incubation medium is shown in Fig. 4, where 0.7ΔpH/0.1ml/hr. of enzymatic hydrolysis of acetylcholine is eaqual to 30 μ mole/0.1ml/hr. in Fig. 4.

FIG. 4 STANDARD CURVE FOR TRANSVALUATION OF THE UNIT OF PH TO μM ACETIC ACID.



Normal range of serum CHE of Wistar-Imamich strain rats determined by this method is shown in Tab. 3 by the unit of ΔpH/hr.

TAB. 3 NORMAL RANGE OF CHE ACTIVITY IN RAT SERUM IN DIFFERENT AGES. (mean±S.D., ΔpH/0.1ml/hr.)

wks. after birth	10	19	26	52
male	0.625±0.101 (92)	0.529±0.129 (95)	0.837±0.125 (15)	0.853±0.165 (16)
female	0.822±0.214 (61)	1.579±0.329 (91)		

( ): Numbers of rats examined

There are some difference between MICAEL's method and this modification. In this modification the incubation temparature is 37°C, but in his method it was 25°C. So the corrections are quite different values from his. Further, MICAEL's corrections seemd to be determined based on the hypothesis that pH_i was constant. But pH_i is variable corresponding to p^H of serum and to CO_2 contents etc. Our survey of this buffer reveals that non-enzymatic hydrolysis depends on pH_i and pH_f, and enzymatic hydrolysis depends on pH_i.

In another ΔpH method of TAKAHASHI & SHIBATA, they do not need corrections. This is supported from our experiment of linearity and nature of b corrections. If the buffer is always made standardly and immediately before determination of CHE, these corrections b and i are not useable.

It is a merit that the values from this method may transvalue to the unit of μ mole/ml/hr. or min. and comparison of the values is possible between ΔpH method and other methods. From this comparison shown in Tab. 4, normal range of rat and human serum CHE is in near level by any method.

TAB. 4 CPMPARISON OF NORMAL RANGE OF CHE ACTIVITY OF HUMAN AND RAT SERUM FROM VARIOUS INVESTIGATORS.

Investigator	Normal range	Unit	Method
human serum			
MANN et al.	0.7-1.6	ΔpH/0.1ml/hr.	MICHAEL
VORHAUS et al.	0.58-1.37	ΔpH/0.1ml/hr.	MICHAEL
DE LA HUERGA et al.	130-310	μ M/ml/hr.	HESTRIN modified
SHIBATA et al.	0.8-1.1	ΔpH/0.1ml/hr.	SHIBATA & TAKAHASHI
MIYAMOTO et al.	1.8-4.2	μ M/ml/min.	BTC
rat serum			
ECOBICHON et al.	m. 0.3 f. 0.7-2.0	μ M/ml/min.	ECOBICHON

CONCLUSION

1. Through the improvement of MICHAEL's method to determine CHE activity, it became clear that non-enzymatic hydrolysis of acetylcholin in the incubation medium depends on pH_i and pH_f, and enzymatic hydrolysis depends on pH_i.

2. Using corrections b and i, stable and precise values of CHE activity has been able to gained. Calculated corrections b and i are shown, former as correction of non-enzymatic hydrolysis, latter as one of enzymatic hydrolysis.

3. Titration of incubation medium with acetic acid brings us comparable values between the units of ΔpH/hr. and μ mole/ml/hr. or min.

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