Bull. Yamaguchi Med. School, Vol. 4, Nos.2,3. 1956

HISTO- AND BIOCHEMICAL STUDIES OF CHOLINESTERASE IN THE HUMAN LIVER TISSUE

IKUZO OHBA

I. Surgical Clinic (Director : Prof. Akira Matsumoto, M. D.) and the Department of Clinichopathology (Director : Prof. Susumu Shibata, M. D.), Yamaguchi Medical School, Ube

(Received November 10, 1956)

The basis of the histochemical study of cholinesterase, an enzyme which hydrolizes acetylcholine, was first laid by *Glick*[1] in 1941, who discovered a specific activity of this enzyme that split the esters of long-chained fatty acids. Based on this principle, *Gomori*[2] devised a technique to stain the enzyme with myristoylcholine. This method was followed by *Koelle's*[3-5] report in which he developed a histochemical method employing acetylthiocholine as substrate. These works stimulated further investigations in this field throughout the world. Here in Japan, reports have been made by *Okinaka*[6] *et al.*, *Toyota*,[7] *Goto*[8] *et al.* and others on the distribution of cholinesterase in various tissues. The present author[9] has also reported on the distribution of this enzyme in human liver tissue as studied by myristoylcholine method.[10] In this communication, further studies of histochemistry in relation to biochemical determination of cholinesterase activity will be discussed.

EXPERIMENTAL

Pretreatment :

A piece of human liver of about one gram was resected and frozen immediately after laparotomy; 10–20μ sections were prepared, extended on cover-glasses and freezing-dried in vacuo. In this cover-glass method, the sections get dried within one minute or two and the stain of cholinesterase is satisfactory, if they are extended on glasses promptly without the help of water. For filter-paper method, 100–200μ frozen sections were prepared and spread on a filter-paper and lyophilized. The remaining portion of the frozen tissue was minced finely and used for biochemical determination.

I. Histochemical Study.

A. Reagents :

1) Cobalt acetate solution : To 500 ml of 0.025 mol barbiturate buffer of pH 7.0 (2.6gm of sod. barbiturate and 11.0 ml 1 n-HCI are diluted to 500 ml

with distilled water) are added 1.6 gm of cobalt acetate ($Co(CH_3COO)_2 \cdot 4H_2O$). To this mixture are added 2.5 ml of 0.2 mol aqueous solutions of each of the following salts; $MgCl_2$, $MnCl_2$ and $CaCl_2 \cdot 6H_2O$.

If tris-maleate buffer is used instead of the above, dissolve 29 gm of maleic acid and 30.3 gm of tris (hydroxymethyl) aminomethane in 500 ml distilled water, add about 2 gm of charcoal, shake, let stand for 10 minutes and filtfier; to 40 ml of this filtrate add 19 ml of n-NaOH and dilute with H_2O to 100 ml, pH 7.0.

2) 0.02 mol aqueous solution of myristoylcholine chloride.

3) Yellow ammonium sulfide-alcohol: Mix 90 ml of 70 per cent ethanol with 10 ml of yellow ammonium sulfide.

B. Procedure:

Incubate the section slides in the mixture of 50 volumes of reagent #1 and 1 volume of #2 for 6–12 hours at 37°C. Wash slides for several seconds in water, immerse them in reagent #3 instead of water for 10–15 minutes to hasten the development of the color. The cover-glass sections are then stained in hematoxylin-eosin. Filter-paper specimens are embedded in paraffin after dehydration; 3–5μ pareffin sections are made and stained with hematoxylin or hematoxylin-eosin. As the blank, dried sections are immersed in the following solution in order to inactivate cholinesterase; 1.0–1.5 ml of 0.1 % eserine are added to 100 ml of the mixture of reagents #1 and #2. For another blank, dried sections are stained simply with hematoxylin to yield untreated control.

C. Results:

Cholinesterase staining of the liver tissue by myristoylcholine method does not always prove successful. Even when the staining seemed satisfactory, the cobalt sulfide granules lacked in homogeneity in distribution, sometimes more densely around the central veins or in the periphery of lobules, rendering the stain uneven. Figures 1–3 are the sections which the author believes most successfully stained. Cholinesterase is distributed along the liver cell cords, more markedly in the central and the peripheral areas of the lobules, whereas the granules are not seen at all in the periportal spaces or in the epithelial cells of the bile ducts. It is illustrious in Fig. 2 and 3 that cobalt sulfide granules are outside the hematoxylin stained nuclei, and that Kupffer cells lack in cholinesterase. Attention has to be drawn to a phenomenon that sometimes a minute amount of brownish yellow granules resembling the real ones appear in the blank and in the untreated control. The illustrative photos demonstrate the real granules derived from cholinesterase, since they exceed the non-specific granules in number as well as in size.

Toyota[7] employing acetylthiocholine method reported that cholinesterase does not exist in protoplasma of the liver cells, but is demonstrable in nuclei and to a

certain extent in the nuclei of epithelial cells of the bile ducts. According to his improved method,[7] cholinesterase was demonstrated in protoplasma of the liver cells but it was not always in the nuclei. He also mentioned that cholinesterase would probably not be demonstrated in Kupffer cells, endothelial cells of veins and arteries of the portal triads or in epithelial cells of the bile ducts. Similar findings have been described by *Goto* and *Aoyagi*.[8] Although the present author's technique was different from theirs, the findings were similar.

II. Biochemical Study.

There have been several methods for the measurement of cholinesterase activity, such as biochemical assay, titrimetric, manometric and colorimetric methods and determination of pH. For the clinical laboratory routine, *Takahashi-Shibata's* technique[11)12)] measuring pH with an indicator is very simple and practicable. This method, however, is not quite applicable to the liver tissue with the same accuracy and sensitivity. Therefore, the author has modified the method of *De La Huerga*,[13)] a modification of *Hestrin's*[14)] technic which is based on hydrolysis of acetylcholine.

A. Reagents:

1) Buffer solution: Mix 13 ml of 0.25 mol citric acid and 187 ml of 0.5 mol Na_2HPO_4, pH 7.6.

2) 14 g/dl hydroxylamine chloride solution: The solution is nearly 2 mol. It can be used for 1 week, when kept in ice-box.

3) 3.5 n-NaOH.

4) Alkaline hydroxylamine solution: Before use, mix the same volumes of reagent #2 and #3.

5) 10 g/dl trichloracetic acid.

6) 5 g/dl acetylcholine solution (concentration has to be exact).

7) 1 n-HCl.

8) Eserine solution: 0.05 g/dl eserine sulfate. Keep in ice-box.

9) $FeCl_3$ solution: To 10 ml of 10 g/dl $FeCl_3 \cdot 6H_2O$ add 1.0 ml of 1 n-HCl, dilute to 100 ml.

B. Procedure:

1) Frozen and minced liver tissue is weighed accurately on the chemical balance, diluted tenfold with saline and homogenized in a glass homogenizer while being cooled.

2) Sample and reagents are taken as follows:

Tube	Homogenate* (ml)	H_2O (ml)	Buffer (ml)	Acetylchol. Solution (ml)
A	0.5	—	0.25	0.25
A'**	0.5	—	0.25	0.25
B (Blank)	—	0.5	0.25	0.25

* In case the liver of dog was used 0.2 ml of homogenate because of high cholinesterase activity. In the case of serum, 0.1 ml was used for human and dog materials.

** To tube A′ add one drop of eserine before the addition of acetylcholine in order to block cholinesterase activity.

3) The whole rack of tubes is then immersed in a water-bath at 37°C. Tubes are taken out exactly after 60 minutes, and one or two drops of eserine are added to tubes A and B.

4) Add 1.0 ml alkaline hydroxylamine solution to each tube, mix, let stand for 3 minutes, add 3.0 ml of 1 n-HCI and 5.0 ml of 10 g/dl trichloracetic acid and shake.

5) Filter A and A′. B is clear and need not be filtered.

6) Prepare tubes *a*, *a′*, *b* and *c* as follows:

Tubes	Filtrate of (ml)	diluted $FeCl_3$ (ml)
a	A 1.0	10.0
a′	A′ 1.0	10.0
b	B 1.0	10.0
c	1 n-HCl 1.0	10.0

7) Let stand for ten minutes for complete color development, which is stable thereafter. Measure the optical density at 500 mμ using *c* as the blank. Let the optical density of *a*, *a′* and *b* be α, α' and β.

C. Standard Curve:

1) 0.1 g/dl acetylcholine solution is measured into tubes as follows:

Tubes	A	B	C	D	E	F
0.1 g/dl acetylchol. (ml)	0.00	0.25	0.50	0.75	1.00	1.25
H_2O (ml)	1.00	0.75	0.50	0.25	0.00	0.00
Alk. hydroxylamine (ml)	1.0	1.0	1.0	1.0	1.0	1.0
..... Let Stand for 3 minutes ..... then add						
1 n-HCI (ml)	3.00	3.00	3.00	3.00	3.00	2.75
10 g/dl Trichl. acet. acid (ml)	5.0	5.0	5.0	5.0	5.0	5.0

2) Take 1.0 ml from each tube and add 10.0 ml of $FeCl_3$ solution. Measure the optical density in the same way with A as the blank.

3) The standard curve is drawn by plotting the following; A=0, B=2.5, C=5.0, D=7.5, E=10.0, F=12.5 mg. The μ Mol concentration is calculated by multiplying the values obtained from the curve by 5.5.

D. Calculation:

Optical densities *a*, *a′* and *b* are converted into acetylcholine amounts from standard curve. In formulae

$$\alpha' - \alpha = \Delta E, \qquad \beta - \alpha' = \Delta E'$$

ΔE represents cholinesterase activity and $\Delta E'$ activity of enzymes other than cholinesterase, each hydrolizes acetylcholine. The cholinesterase activity per unit-amount of tissue or serum is computed from the dilution factor and expressed in terms of μ Mol per gram or ml.

E. Results:

Cholinesterase activities measured by the described method in the liver tissue and the histochemical findings are given in Table 1. The marks (−)−(+++) denote the number of granules per cell; (−) none, (+) 1 to several, (++) several to 10, and (+++) more than 10 granules. The degree (+++) of granules is typically shown in Fig. 1–3.

TABLE 1

Patient	Sex	Age	Diseases	Cholinesterase activity of the human liver		
				Chemical (μMol/g)	Histochemical	
					Periphery of lobules	Central regions
Y.M.	♂	55	Gastric cancer	137.5	−	+
S. I.	♀	24	Banti's syndrome	242.0	+	+
T. F.	♂	30	Gastric ulcer	220.0	++	−
H.K.	♂	47	Chronic gastritis	187.0	+	+
K.N.	♂	52	Gastric polyp	231.0	++	−
C.O.	♀	48	Gastric cancer	209.0	−	+
T.Y.	♂	48	Gastric ulcer	165.0	+	−
S. K.	♂	55	Cholecystitis	209.0	−	+
H. S.	♂	61	Esophageal cancer	198.0	+	−
S. K.	♂	67	Gastric cancer	198.0	+	+
M.Y.	♀	50	Cholecystitis	165.0	−	−
M.K.	♀	39	Gastric cancer	176.0	+++	+++
S. T.	♂	25	Pyloric ulcer	154.0	+	++
K.F.	♂	60	Gastric ulcer	93.5	++	+
K.N.	♂	72	Liver cirrhosis	115.0	+	−
M. I.	♂	26	Pyloric ulcer	132.0	−	++
Y.Y.	♂	50	Cholelithiasis	165.0	−	+
T.M.	♂	46	Banti's syndrome	71.5	+	−

DISCUSSION

There have been reported a number of methods for biochemical and histochemical determinations of cholinesterase and studies on this enzyme using such techniques. Since cholinesterase is an enzyme, the procedure for the quantitative demonstration involves many limiting factors and even a slight inconsistency of the technique brings about poor results. The same technical conditions do

not always guarantee reproducible results, just like the cases of other tissue stainings. The inconsistency of the result was further confirmed by employing acetylthiocholine as substrate according to *Toyota's* method, which is a modfication of the *Koelle's*. The detail is not reported here because of the insufficient supply of this compound and thereby not enough data to discuss with. For this reason, several to more than ten slides were always prepared for one specimen and only successfully stained ones were tabulated and evaluated in this paper.

As will be clearly noticed from Table 1, there is a wide discrepancy between biochemical and histochemical determinations of cholinesterase. It does not appear feasible to correlate both data obtained by two different methods. In the present study the objective was mainly to demonstrate nonspecific cholinesterase rather than the specific one, as based on the description of *Gomori*[10] and *Toyota*[7] etc., that cholinesterase in the liver is not specific. Such discrepancy may not be ensued by specific cholinesterase, if it may exist. It therefore has little meaning to discuss the enzyme with the stained sections on the quantitative basis. Nevertheless, the histochemical method is the only technique available for the study of the distribution of the enzyme. The specimen treated with the coverglass method is too thick for differential evaluation of individual cell. In order to eliminate such a shortcoming Shen[15] et al. used a paraffin section method, which, however, was not very satisfactory because of possible inactivation of the enzyme during the pretreatment. On the other hand, the author's filter-paper technique involves very little interference with the enzyme activity, because the paraffin sections were prepared after the staining. Therefore observations of individual cell are possible. As the substrate does not infiltrate deep into the tissue with this method, only two or three sections are obtainable from the surface of the tissue. Cholinesterase in serum and its behavior in various diseases will be reported elsewhere.

CONCLUSION

Cholinesterase activity was studied in the liver tissue with a filter-paper technique developed by the author as based on Gomori's myristoylcholine method. The enzyme was demonstrated only inside the protoplasma of the liver cells, but not in their nuclei, Kupffer cells, periportal spaces or in the epitherial cells of the bile ducts. The biochemical determination of this enzyme performed in parallel with the histochemical study disclosed a very poor correlation between the degree of stain and the chemically determined activity. It was, therefore, suggested that the histochemical study of the sections does not always provide basis for the quantitative evaluation.

Grateful acknowledgement the author wishes to make to Professor Akira Matsumoto, Professor Susumu Shibata and Dr. Hiroshi Takahashi for their kind

advices during this study.

REFERENCES

1) GLICK, D.: Some Additional Observations on the Specifity of Cholinesterase. *J. Biol. Chem.* **137**: 375-362, 1941.
2) GOMORI, G. : Histochemical Determination of Sites of Cholinesterase Activity. *Proc. Soc. Exper. Biol. & Med.*, **68** : 354-358, 1948.
3) KOELLE, G. B. and FRIEDENWALD, J. S. : A Histochemical Method for Localizing Cholinesterase Activity. *Proc. Soc. Exp. Biol. & Med.* **70** : 617- 622, 1949.
4) KOELLE, G.B.: The Histochemical Differentiation of Types of Cholinesterases and their Localizations in Tissues of the Cat. *J. Pharm. Exp. Therap.*, **100** :158-179, 1950.
5) KOELLE, G. B.: The Elimination of Enzymatic Diffusion Artifacts in the Histochemical Localization of Cholinesterases and a Survey of their Cellular Distributions. *Ibid.*, **103**: 153-171, 1951.
6) OKINAKA, S., et al : Biochemical Studies on Action of the Nerve. VII. Histochemical Determination of Cholinesterase. *Igaku to Seibutsugaku* (Medicine and Biology), **26** : 221-224, 1953.
7) TOYOTA, M.: Histochemistry of Cholinesterase. Report I. *Nisshin Igaku* (Jap. J. Med. Prog.), **40** : 559-568, 1953.
8) GOTO, J. and AOYAGI, Y.: Histochemical Studies on Cholinesterase in the Stomach, the Intestine and the Liver. *Igaku to Seibutsugaku* (Medicine and Biology), **29** : 98-102, 1953.
9) OHBA, I. : Distribution of Cholinesterase in the Human Liver Tissue. *Igaku to Seibutsugaku* (Medicine and Biology), **33** : 149-152, 1954.
10) GOMORI, G. : *Microscopic Histochemistry*. University of Chicago Press. Chicago, 1952.
11) TAKAHASHI, H. and SHIBATA, S. : A Simple Method for the Serum Cholinesterase Determination Applicable to Routine Examination. *Igaku to Seibutsugaku* (Medicine and Biology), **20** : 96-98, 1951.
12) TAKAHASHI, H. : Studies on Serum Cholinesterase. I. A Method for the Estimation of Serum Cholinesterase Activity which is Useful in the Routine Work of Clinical Laboratory. *Bull. Yamaguchi Med. Sch.*, **3** : 155-165, 1956.
13) DE LA HUERGA, J., YESINICK, C., and Popper, H. : Colorimetric Method for the Determination of Serum Cholinesterase. *Am. J. Clin. Path.*, **22** : 1126-1133, 1952.
14) HESTRIN, S.: The Reaction of Acetylcholine and other Carboxylic Derivatives with Hydroxylamine and its Analytical Application. *J. Biol. Chem.*, **180** :249, 1949.
15) SHEN, S. C. et al. : Application of Histochemical Technic for Cholinesterase to Paraffin Sections. *Proc. Soc. Exp. Biol. & Med.*, **81** : 452-455, 1952.

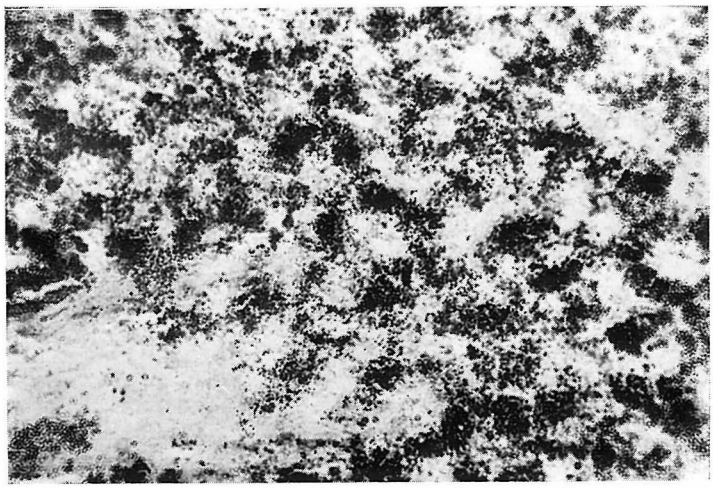

Fig. 1. Distribution of cholinesterase in the human liver tissue (by cover-glass method). × 100.

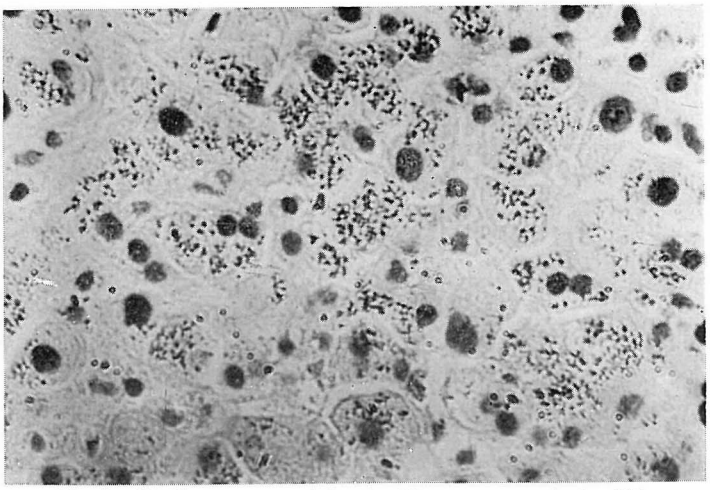

Fig. 2. Cholinesterase staining by filter-paper method, × 200.

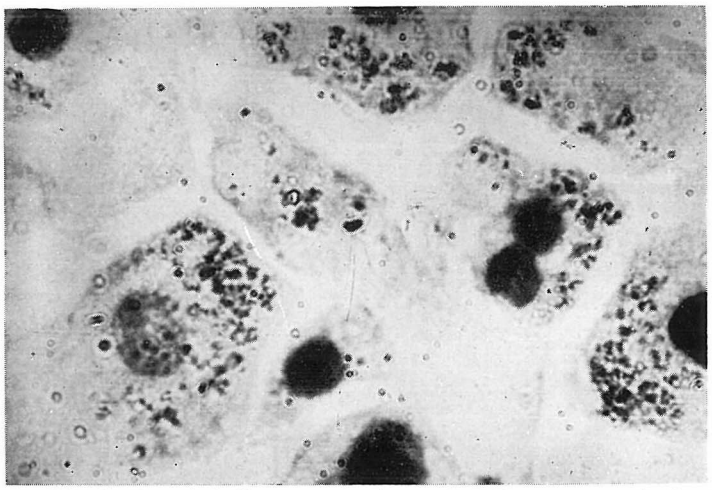

Fig. 3. Distribution of cholinesterase in liver cells (Filter-paper method), × 1000.