Acetylcholinesterase

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The enzyme acetylcholinesterase (AChE) which is highly specific and only hydrolyses acetylcholine, is distinct from cholinesterase (ChE) which splits other esters as well as acetylcholine, *e.g.* butyrylcholine, benzoylcholine, succinylcholine, tributyrin and similar esters[1]. AChE is often called "true cholinesterase". It is mainly found in the brain, in nerve cells (especially end plates), in muscle (the amount depends on the number of nerve endings) and in erythrocytes. The enzyme is also widely distributed in the animal kingdom. For example, 400 to 500 mg. of chicken brain can hydrolyse 10^{14} molecules acetylcholine in a millisecond[2]. The electric organ of the electric ray *(Torpedo vulgaris)* is one of the richest sources of AChE[3].

Cholinesterase occurs mainly in human serum, where it was first discovered[4]. Recently a specific AChE has been demonstrated in serum, which differs from the AChE occurring in erythrocytes[5]. After electrophoretic separation of the serum proteins the activity of acetylcholinesterase occurs in a different fraction from the activity with tributyrin and butyrylcholine[6]. The pH optimum of the AChE of serum and erythrocytes is around pH 7.2. However, both enzymes have a distinctly different optimum substrate concentration[5, 7].

The measurement of serum AChE activity is of diagnostic value because an alteration of the normal value occurs with various diseases[8], for example, in hepatitis[9, 10], in patients with malignant tumours[10, 11], in bronchial asthma[12] and in pulmonary tuberculosis[13, 14]. The AChE activity in serum is altered by sex hormones[15], and also by several drugs (*e.g.* sympatol, atropine, physostigmine, prostigmine, cocaine, muscarine, *etc.*.)[16], while the activity of muscle AChE is influenced by testosterone and insulin[17].

The most important methods for the determination of AChE activity are based on the following principles: Manometric measurement[18, 19] of carbon dioxide liberated from bicarbonate by the acetic acid formed enzymatically; electrometric measurement of the change in pH due to the acetic acid formed[20] (for convenience this pH change can be measured with an indicator[21]); titration of the acetic acid (*e.g.* [22]); spectrophotometric measurement of the acetic acid as the ferric acetate complex[23];

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1) *K. B. Augustinsson* in *D. Glick:* Methods of Biochemical Analysis. Interscience Publishers, New York 1957, Vol. 5, p. 2.
2) *D. Nachmansohn*, C. R. Séances Soc. Biol. Filiales Associées *127*, 670 [1938].
3) *D. Nachmansohn* and *N. Lederer*, C. R. Séances Soc. Biol. Filiales Associées *130*, 321 [1939].
4) *E. Stedman* and *L. H. Easson*, Biochem. J. *26*, 2056 [1932].
5) *W. Pilz*, Z. ges. exp. Med. *132*, 310 [1959].
6) *W. Pilz*, unpublished results.
7) *W. Pilz*, Klin. Wschr. *36*, 1017 [1958].
8) *L. J. Vorhaus* and *R. M. Kark*, Amer. J. Med. *14*, 707 [1953].
9) *L. J. Vorhaus*, *H. S. Scudamore* and *R. M. Kark*, Amer. J. med. Sci. *221*, 140 [1951].
10) *F. Zinnitz* and *H. Enzinger*, Münchener med. Wschr. *1950*, 1170.
11) *H. J. Wetstone*, *R. V. LaMotta*, *A. Belluci*, *R. Tennant* and *B. V. White*, Ann. intern. Med. *52*, 102 [1960].
12) *H. S. Scudamore*, *L. J. Vorhaus* and *R. M. Kark*, J. Lab. clin. Med. *37*, 860 [1951].
13) *H. Münch* and *E. Auenmüller*, Beitr. Klin. Tuberkul. *109*, 482 [1953].
14) *H. Hörlein*, Tuberkulosearzt *4*, 512 [1950].
15) *K. Habbe* and *W. Pförtner*, Dtsch. med. Wschr. *76*, 269 [1951].
16) *E. Keeser*, Klin. Wschr. *17*, 1811 [1938].
17) *A. N. Granitsas*, *N. Dede* and *A. J. Philippu*, Nature [London] *185*, 320 [1960].
18) *K. B. Augustinsson*, Acta physiol. scand. *15*, Suppl. 52, 1 [1948].
19) *K. B. Augustinsson*, Acta physiol. scand. *35*, 40 [1955].
20) *H. O. Michel*, J. Lab. clin. Med. *34*, 1564 [1949].
21) *J. Gregoire* and *N. Limozin*, Bull. soc. Chim. biol. *37*, 66, 81 [1955].
22) *O. G. Cesaire*, Ann. Biol. clin. [Paris] *10*, 84 [1952].
23) *N. O. Abdon* and *B. Monäs*, Scand. Arch. Physiol. *76*, 1 [1937].

use of substrates yielding a phenol on hydrolysis, which can be coupled with a diazotized amine and the azo-dye measured[24, 25]; UV measurement of the substrate removed (esterified and non-esterified substrates have different absorption maximum[26]); the determination of residual acetylcholine (usually spectrophotometric) which has been added in excess[27, 28].

The spectrophotometric microdetermination of esters, especially acetic acid esters, has recently been re-examined and the errors present in the earlier methods (especially[28, 29]) have been corrected[30]. On the basis of these results a spectrophotometric micromethod for the determination of AChE activity in human serum and erythrocytes has been described[7]. This method has proved to be by far the best for the clinical laboratory. It requires only small amounts of biological material, it is rapid, and the accuracy is at least equivalent, if not superior, to all other methods.

Principle

Acetylcholinesterase (AChE) catalyses the reaction:

$$\text{Acetylcholine} + H_2O \longrightarrow \text{choline} + \text{acetic acid}$$

Biological material is incubated with excess acetylcholine, after a measured period the incubation is stopped and the residual acetylcholine is determined spectrophotometrically according to[30]. This method can be used without alteration for the determination of AChE in any biological material.

Optimum Conditions for Measurements

The pH optimum of AChE is around pH 7.2[7]. Under the conditions of the method described here the optimum concentration of acetylcholine is between 25 to 50 μequiv. in 20 ml. solution (incubation volume) for 0.2 ml. serum[5]. In order to work at the optimum substrate concentration for the determination of AChE in erythrocytes, the erythrocytes from 0.2 ml. of whole blood must be taken[6].

In studies on biological material other than human serum and erythrocytes, the optimum substrate concentration is not always reached. As long as the conditions given below are adhered to this does not greatly interfere and the results are comparable one with another. Little attention has been paid in previously published methods to the optimum concentration of substrate.

Reagents

1. Sodium hydroxide, A. R., 1 N; 2.5 N and 32% (w/v)
2. Hydrochloric acid, A. R., 1 N and conc.
3. Sodium chloride, A. R.
4. Acetic acid, 1 N
5. Citric acid·1 H_2O
6. Boric acid, H_3BO_3
7. Hydroxylamine hydrochloride
8. Ferric ammonium sulphate, $FeNH_4(SO_4)_2 \cdot 12H_2O$
9. Potassium nitrate, KNO_3
10. Trichloroacetic acid

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24) *K. A. Ravin, K. C. Tsou* and *A. M. Seligman*, J. biol. Chemistry *191*, 843 [1941].
25) *D. N. Kramer* and *R. M. Gamson*, Analytic. Chem. *30*, 251 [1958].
26) *W. Kalow* and *H. A. Lindsay*, Canad. J. Biochem. Biophysics *33*, 568 [1955].
27) *S. Hestrin*, J. biol. Chemistry *180*, 249 [1949].
28) *R. L. Metcalf*, J. econ. Entomol. *44*, 883 [1951].
29) *J. H. Fleisher* and *E. J. Pope*, Arch. ind. Hyg. occupat. Med. *9*, 323 [1954].
30) *W. Pilz*, Z. analyt. Chem. 162, 81 [1958].

11. Acetylcholine hydrochloride *)
12. Saponin album *)
13. Prostigmine **)

Preparation of Solutions

I. Citrate buffer (1 M; pH 1.4):
In a 500 ml. volumetric flask dissolve 105 g. citric acid·1 H_2O and 4.0 g. NaOH in a little distilled water, add 445 ml. 1 N HCl, dilute with distilled water to the mark. Measurement of pH: dilute 10 ml. buffer with distilled water to 100 ml., measure with a glass electrode.

II. Saponin (0.1% w/v):
In a volumetric flask dissolve 1 g. saponin album in distilled water and make up to 1000 ml.

III. Prostigmine (5 μg./ml.):
Dilute the contents of a prostigmine ampoule (1 ml.; 0.5 mg.) with saponin solution (II) to 100 ml.

IV. Acetate buffer (0.2 M; pH 4.62):
Dilute 200 ml. 1 N acetic acid + 100 ml. 1 N NaOH to 1000 ml. with distilled water.

V. Borate solution:
Dissolve 31.0 g. boric acid in *ca.* 700 ml. distilled water, add 20 ml. 1 N NaOH and dilute with distilled water to 1000 ml.

VI. Acetylcholine:
a) Stock solution (0.016 M; pH 4.62):
In a 500 ml. volumetric flask dissolve 1.4532 g. acetylcholine chloride in 50 ml. acetate buffer (solution IV) and dilute to 500 ml. with distilled water.
b) Substrate solution (0.0032 M; pH 7.2):
In a 50 ml. volumetric flask dilute 10 ml. acetylcholine stock solution (VIa) with 10 ml. borate solution (V) and dilute to 50 ml. with distilled water.

VII. Hydroxylamine (1 N):
Dissolve 70 g. hydroxylamine hydrochloride in distilled water and make up to 1000 ml.

VIII. Iron (ferric) (0.7 M):
Dissolve 337.5 g. $FeNH_4(SO_4)_2 \cdot 12H_2O$ and 25 g. potassium nitrate in distilled water and make up to 1000 ml.

IX. Trichloroacetic acid (50% w/v):
Dissolve 500 g. trichloroacetic acid in *ca.* 300 ml. distilled water, add a little 0.1% solution of phenolphthalein in alcohol, neutralize exactly with 32% (w/v) NaOH with cooling and remove the resulting red colour with a drop of conc. HCl. Dilute to 500 ml. with distilled water.

X. Physiological saline (0.9% w/v NaCl):
Dissolve 9 g. NaCl in distilled water and make up to 1000 ml.

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*) *e.g.* from E. Merck, Darmstadt (Germany).
**) *e.g.* from Deutsche Hoffmann-La-Roche, AG., Grenzach/Baden (Germany); in 1 ml. ampoules each containing 0.5 mg prostigmine.

Stability of the solutions

The saponin and prostigmine solutions (II and III) are stable for 4–6 weeks in a refrigerator. The acetylcholine stock solution (VIa) keeps for *ca.* 1 month in a refrigerator, the dilute substrate solution (VIb) must be freshly prepared for each series of determinations (stable 3–4 hours). Do not keep the hydroxylamine solution (VII) for longer than 14 days.

Procedure

Preparation of the experimental material

Serum: In a 25 ml. volumetric flask dilute 1.0 ml. of serum, free from haemoglobin, with physiological saline (X) to the mark.

Erythrocytes: Into a centrifuge tube containing *ca.* 5 ml. physiological saline (X), pipette with vigorous shaking 1.0 ml. fresh whole blood, shake and centrifuge 10 min. at 3000 r.p.m. Discard supernatant, add a further *ca.* 5 ml. physiological saline (X), mix well and centrifuge again. Repeat this process four times. On decanting the supernatant fluid, which should always appear colourless, be careful that no erythrocytes are poured off. Wash out the residual erythrocytes into a 25 ml. volumetric flask with saponin solution (II) and dilute to the mark with the same solution. Mix well.

Other biological material: Either dissolve the sample in saponin solution (II) or after addition of physiological saline (X) emulsify as finely as possible in a homogenizer and filter. Take care that no organic solvents (*e.g.* alcohol) come into contact with the biological material as they inhibit the enzyme. Dilute the filtrate with sufficient physiological saline (X) so that during the incubation no more than a quarter of the added acetylcholine is hydrolysed.

Incubation mixture

For every determination a control is prepared, because during the period of incubation an appreciable spontaneous hydrolysis of the substrate occurs and occasionally the experimental material is coloured. This control contains substrate and sample as in the determination, but AChE is completely inhibited by the addition of prostigmine.

A reagent blank is prepared in addition to the determination (in duplicate) and control. For each estimation four 50 ml. volumetric flasks are required.

Pipette successively into the flasks:

	Control	*Determinations*	*Reagent blank*
prostigmine solution (III)	5 ml.	—	—
saponin solution (II)	—	5 ml.	—
sample solution	5 ml.	5 ml.	—
Allow to stand 10 min. at room temperature, then add substrate solution (VIb)	10 ml.	10 ml.	—

Incubate 30 min. at 37°C (incubator or thermostatically controlled water bath); during this time mix 20 ml. hydroxylamine solution (VII) and 20 ml. 2.5 N NaOH ("alkaline hydroxylamine solution").

After incubation, pipette into the flasks:

alkaline hydroxylamine solution	5 ml.	5 ml.	5 ml.
prostigmine solution (III)	—	5 ml.	—
buffer (solution I)	5 ml.	5 ml.	5 ml.
Fe^{3+} solution (VIII)	10 ml.	10 ml.	10 ml.
trichloroacetic acid (IX)	5 ml.	5 ml.	5 ml.

Dilute all the flasks (control, determinations and reagent blank) to the mark with water, shake well and allow to stand for 15 min. in a place protected from direct sunlight. Filter through a double fluted filter paper (Schleicher & Schüll No. 602h) into a dry 50 ml. Erlenmeyer flask. Discard the first portion of the filtrate.

Immediately afterwards measure spectrophotometrically at 490 mμ (or corresponding filter) against reagent blank in cuvettes with a 1 cm. light path.

Calculations

The enzyme activity is given in μequiv. acetylcholine hydrolysed under the standard conditions of the method.

The optical densities obtained for the two determinations are averaged. The amount of acetylcholine hydrolysed enzymatically is obtained from the difference in optical density between the control and determination.

$(E_{control} - E_{determination}) \times 52$ = μequiv. of acetylcholine hydrolysed enzymatically by the added sample.

The factor 52 is obtained from the molar extinction coefficient for the ferri-complex of acetohydroxamic acid[30], the total volume of the assay mixture (50 ml.) and the light path used (1 cm.).

The maximum error with 1000 estimations amounted to ± 4%.

Example

The following optical densities were obtained with serum: Control: 0.612; determination$_1$: 0.482; determination$_2$: 0.478; therefore it follows:

$$\left(0.612 - \frac{0.482 + 0.478}{2}\right) \times 52 = 6.86 \text{ μequiv. acetylcholine hydrolysed}$$

The AChE activity of the serum is 6.86 μequiv. acetylcholine hydrolysed.

Conversion to other units

a) Conversion to μequiv./hour (corresponding to the amount of sample added): multiply the result [μequiv.] by 2 [μequiv.]

b) Conversion to μl. CO_2/hour (corresponding to the amount of sample added): multiply the result by 44.8

Stability of the Enzyme in Samples of Biological Material

Serum diluted with physiological saline and haemolysed erythrocytes in saponin solution can be stored for at least four days between 2 and 6°C without a detectable loss of enzyme activity. The same is true for extracts from other biological material.

Sources of Error

The following substances cause interference: oxidizing and reducing agents, and organic solvents (alcohol, ether, acetone, *etc.*).

Special care should be taken to ensure that the complete analysis is carried out in the absence of direct sunlight, because even a short exposure leads to large errors. Light from tungsten bulbs or fluorescent tubes does not interfere; UV radiation causes considerable interference.

Influence of Therapeutic Agents

Eserine[31] and prostigmine are inhibitors of AChE, the latter being the more active[32]. Phosphoric acid esters, which are used partly as insecticides (*e.g.* Meta-Systox-i and E-605)[33] irreversibly inhibit AChE. This inhibition occurs both *in vivo*[34] and *in vitro*[6]. Since these phosphoric acid esters are widely used as plant protection agents, the determination of AChE activity is of importance in the diagnosis of poisoning by these agents.

Pyridine-2-aldoxime-iodomethylate[35] causes partial or complete reactivation of AChE inhibited by phosphoric acid esters[36]. For the therapeutic use of this preparation refer to[37,38]. The *in vitro* use of this preparation for diagnosis of phosphoric acid ester poisoning (refer to[39]) is not yet sufficiently certain.

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31) *L. Loewi* and *N. Navratil*, Pflügers Arch. ges. Physiol., Menschen Tiere *214*, 678 [1926].
32) *R. Ammon*, Zbl. inn. Med. *63*, 114 [1942].
33) *G. Schrader:* Die Entwicklung neuer Insektizide auf Grundlage organischer Fluor- und Phosphorverbindungen. Verlag Chemie, Weinheim/Bergstr. 1952.
34) *S. Davidson*, Biochem. J. *61*, 203 [1955].
35) *I. B. Wilson* and *S. Ginsburg*, Biochem. biophysica Acta *18*, 168 [1955].
36) *F. Hobbiger*, Brit. J. Pharmacol. *11*, 295 [1956].
37) *W. D. Erdmann*, *F. Sakai* and *F. Scheler*, Dtsch. med. Wschr. *83*, 1359 [1958].
38) *W. D. Erdmann*, Dtsch. med. Wschr. *95*, 1014 [1960].
39) *R. Schön* and *H. Südhof:* Biochemische Befunde in der Differentialdiagnose innerer Krankheiten. Georg Thieme Verlag,Stuttgart 1959.