Cholinesterases

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With the enzymes which hydrolyse acetylcholine, distinction must be made between a true *acetylcholinesterase* and an unspecific *cholinesterase*. According to *Augustinsson*[1] there is a considerable difference between the two types:

	Cholinesterase I, specific cholinesterase, true cholinesterase, e-type	Cholinesterase II, unspecific cholinesterase, pseudocholinesterase, s-type
Main source	erythrocytes, nerve tissue, thymus, *etc.*	serum, pancreas, glands
Optimum pH	7.5–8.0	8.5 *)
Isoelectric point	4.65–4.70	4.36
Stability to variations in pH	low	high
Inhibition by excess substrate	+	–
Activation by NaCl	+	–
Hydrolysis of:		
Tributyrin	–	+
Acetyl-β-methylcholine	+	–
Benzoylcholine	–	+
Phenylacetylcholine	–	+
Atrolacetylcholine	–	+

The decrease in unspecific cholinesterase activity in serum, which occurs with damage to the liver parenchyma or in intoxication of organic phosphates, is of clinical importance.

Numerous assay methods have been developed; mainly colorimetric (I), manometric (II) and titrimetric (III):

I. Determination of the acetic acid liberated with $FeCl_3$[2]; formation of acetylhydroxamic acid from the residual acetylcholine with alkaline hydroxylamine solution[3].

II. Determination of the rate of hydrolysis with the *Warburg-Barcroft* or *Van Slyke* apparatus[4–6].

III. Titration of the acid produced, either potentiometrically[7] or by means of indicators[8].

IV. Histochemical assay[9] by the thiol analysis of acetylthiocholine (see p. 937).

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*) The optimum pH according to *Hestrin*[3] is 8.2. The gasometric methods are carried out at pH 7.3.

1) *K. B. Augustinsson*, Acta physiol. scand. *15*, Suppl. 52 [1948].
2) *N. O. Abdon* and *B. Uvnäs*, Scand. Arch. Physiol. *76*, 1 [1937].
3) *S. Hestrin*, J. biol. Chemistry *180*, 249 [1949].
4) *R. Ammon*, Pflügers Arch. ges. Physiol. Menschen Tiere *233*, 486 [1933].
5) *E. Stedman* and *E. Stedman*, Biochem. J. *29*, 2107 [1935].
6) *M. Rinkel* and *M. Pijoan*, J. Pharmacol. exp. Therap. *64*, 228 [1938].
7) *D. Glick*, J. gen. Physiol. *21*, 289 [1938].
8) *E. Stedman*, *E. Stedman* and *L. H. Easson*, Biochem. J. *26*, 2056 [1932].
9) *G. B. Koelle* and *J. S. Friedenwald*, Proc. Soc. exp. Biol. Med. *70*, 617 [1949].

A. Colorimetric Method

Principle

Cholinesterase catalyses the hydrolysis of esters where the alkyl and acyl groups have certain specific configurations:

If acetylcholine is used as substrate, then the unhydrolysed acetylcholine can be converted with hydroxylamine to acetylhydroxamic acid, which can be determined as the brown ferric complex:

The method given below is a modification by *Huerga, Yesinick* and *Popper*[10] of the original method of *Hestrin*[3].

Optimum Conditions for Measurements

For the cholinesterase of serum the following are optimal: acetylcholine 4.5×10^{-2} M, 2.5×10^{-3} M K^+, 4×10^{-2} M Mg^{2+} and pH 8.2.

Reagents

1. Diethylbarbituric acid (veronal), sodium salt
2. Hydrochloric acid, A. R., 1 N, 0.5 N and 0.02 N
3. Sodium carbonate, anhydrous, A. R.
4. Magnesium chloride, anhydrous, A. R.
5. Potassium chloride, A. R.
6. Acetylcholine chloride*)
   recrystallized from 96% ethanol.
7. Hydroxylamine hydrochloride
8. Sodium hydroxide, A. R.
9. Ferric chloride, $FeCl_3 \cdot 6\,H_2O$

Preparation of Solutions

I. Veronal buffer (0.1 M; pH 8.2):
Dissolve 10.3 g. Na veronal in 300 ml. distilled water and slowly add 60 ml. 1 N HCl; this causes crystals to form. Add 5.3 g. sodium carbonate and, while heating, stir until all the crystals disappear. Allow to cool to room temperature and dilute to 500 ml. with distilled water.

II. Salt solution (0.44 M $MgCl_2$; 0.03 M KCl):
Dissolve 4.2 g. $MgCl_2$ and 0.2 g. KCl in distilled water and make up to 100 ml.

III. Acetylcholine (0.5 M):
Dissolve 910 mg. acetylcholine chloride in distilled water and make up to 10 ml.

*) Use the commercially available acetylcholine chloride (ampoules of dry acetylcholine chloride from Hoffman-La Roche). The bromide or iodide of acetylcholine are less hygroscopic.

10) *J. de la Huerga, Ch. Yesinick* and *H. Popper*, Amer. J. clin. Pathol. *22*, 1126 [1952].

IV. Acetylcholine-buffer-salt mixture:
Just before use mix 8 volumes of veronal buffer (solution I), 1 volume of acetylcholine solution (III) and 1 volume of salt solution (II).

V. Hydroxylamine (14% w/v $NH_2OH \cdot HCl$):
Dissolve 14 g. $NH_2OH \cdot HCl$ in distilled water and make up to 100 ml.

VI. Sodium hydroxide (14% w/v):
Dissolve 14 g. NaOH in distilled water and make up to 100 ml.

VII. Alkaline hydroxylamine solution:
Just before use mix equal parts of solutions V and VI.

VIII. Ferric chloride (1% w/v $FeCl_3 \cdot 6\ H_2O$):
Dissolve 10 g. $FeCl_3 \cdot 6\ H_2O$ in 1000 ml. 0.02 N HCl.

Stability of the solutions

Prepare solutions I and V freshly each month and store in a refrigerator. Prepare solutions III and VII freshly each time. All the other solutions are virtually stable indefinitely.

Procedure

Enzymatic reaction

Final volume: 2.2 ml.; temperature: 37°C; (constant temperature water bath).
For each series of measurements prepare a control tube containing distilled water instead of serum.

Pipette successively into test tubes:

2.0 ml. acetylcholine-buffer-salt-mixture (IV).

Equilibrate at 37°C and add

0.2 ml. serum (free from haemolysis).

Mix thoroughly and incubate for exactly 1 hour at 37°C.

Colorimetric measurements

Wavelength: 540 mμ; light path: 1 cm.

Pipette into the tubes from the enzymatic reaction:

2.0 ml. hydroxylamine solution (VII).

Mix, after 1 min. add

6.0 ml. 0.5 N HCl

and then shake thoroughly. Acetylhydroxamic acid is formed from the unhydrolysed acetylcholine.

Pipette into 15 ml. centrifuge tubes:

10 ml. ferric chloride solution (VIII)
0.5 ml. hydroxamic acid solution (from sample and control tubes).

Mix thoroughly and centrifuge for 5 min. at *ca.* 2000 r. p. m. Carry out the colorimetric measurements on the supernatant. Obtain the μmoles of acetylcholine from a standard curve.

Standard curve

To prepare the acetylcholine standard curve, dilute 0.8, 1.2, 1.6 and 2.0 ml. solution (IV) (corresponding to 40, 60, 80 and 100 μmoles acetylcholine) with distilled water to 2.2 ml.

and then proceed as described under "Colorimetric measurements". Plot the optical densities (ordinate) against the μmoles acetylcholine (abscissa).

Calculations

According to *Huerga, Yesinick* and *Popper*[10] a cholinesterase unit is the amount of enzyme which, under the experimental conditions given here, hydrolyses 1 μmole acetylcholine/hour. The difference in acetylcholine concentration between the control and the experimental tube, multiplied by 5, gives the enzyme units present in 1 ml. serum.

Normal Values

Normal values for human serum (hydroxamate method) are 130–310 units/ml.[10] (animal sera usually give very different cholinesterase values).

B. Manometric Method

Principle

The amount of acetylcholine hydrolysed can be determined directly with the *Warburg* apparatus. The acetic acid liberated sets free an equivalent amount of CO_2 from a bicarbonate-CO_2 buffer. According to *Ammon* and *Voss*[11] the following method is suitable for serum.

Reagents

1. Sodium chloride, A. R.
2. Potassium chloride, A. R.
3. Calcium chloride, $CaCl_2 \cdot 6\ H_2O$
4. Sodium hydrogen carbonate, A. R.
5. Acetylcholine chloride, see p. 772.

Preparation of Solutions

I. Ringer-bicarbonate solution:
Mix the following solutions: 100 ml. 0.9% (w/v) NaCl, 2 ml. 1.2% (w/v) KCl, 2 ml. 1.76% (w/v) $CaCl_2 \cdot 6\ H_2O$ and 30 ml. 1.26% (w/v) $NaHCO_3$. Before use gas with 5% CO_2 in N_2.

II. Substrate solution (0.5% acetylcholine chloride):
Dissolve 50 mg. acetylcholine chloride in solution (I) and make up to 10 ml.

Stability of the solutions

The Ringer-bicarbonate solution is stable in a refrigerator indefinitely. Prepare the substrate solution (II) freshly each day.

Procedure

Experimental material

Dilute haemolysis-free serum 1 : 50 with Ringer-bicarbonate solution (I).

Manometric measurements

Final volume: 2.0 ml.; temperature: 37°C; gas phase: 5% CO_2 in nitrogen.
For each determination prepare 1–2 controls to measure the spontaneous hydrolysis of acetylcholine (and also a thermobarometer, see p. 30, 40). They contain solution (I) instead of serum.

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[11] *R. Ammon* and *G. Voss*, Pflügers Arch. ges. Physiol. Menschen Tiere *235*, 393 [1935].

Nicolai cups[12] are recommended for the measurements.

Pipette into the main compartment

1.5 ml. substrate solution (II)

and in the side arm

0.5 ml. dilute serum.

Equilibrate for about 10 min. Close the manometer taps and tip the serum into the main compartment. Read the manometer levels at 10 min. intervals for *ca.* 1 hour.

Calculations

Correct the increase in pressure in the vessels with serum (mm. Brodie fluid, see p. 40) for the changes occurring in the control vessel and multiply the corrected values by the flask constants. Plot the μl. CO_2 (abscissa) against the time (ordinate) and obtain the μl. CO_2 produced per hour. Divide this value by 22.4 μl. (volume of 1 μmole of gas) to obtain the μmoles of acetic acid liberated per hour (= μmoles acetylcholine hydrolysed). This value must be multiplied by 100 to obtain the units/ml. (= μmole acetylcholine/hour/ml.), because only 0.01 ml. serum (0.5 ml. of $^1/_{50}$ dilution) is taken for the assay.

Conversion to other units

Owing to the different experimental conditions of the methods interconversion of the units is impossible.

Normal Values

According to *Richter* and *Croft*[13] normal values for human serum are from 90 to 240 units/ml. According to *Ammon* and *Zapp*[14] the normal range is from 140 to 200 units/ml. serum.

Effect of Therapeutic Agents

According to *Abderhalden*[15] the esterase activity in serum is temporarily decreased by oestrogens, cortisone and drugs such as, quinine, atropine, hyoscyamine, morphine, codeine, caffeine, theobromine, theophyllin, barbiturates, antipyrin, sulphanilamide, quaternary ammonium compounds, anti-malarial agents, atoxyl, chloroform, amidon, antihistamines, reserpine, lysergic acid, *etc.*

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[12] *P. Rona* and *H. W. Nicolai*, Biochem. Z. *172*, 82 [1926].
[13] *D. Richter* and *P. G. Croft*, Biochem. J. *36*, 746 [1942].
[14] *R. Ammon* and *F. J. Zapp*, Klin. Wschr. *33*, 759 [1955].
[15] *R. Abderhalden:* Klinische Enzymologie. Georg Thieme, Stuttgart 1958.