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Ion-selective Electrodes in the Study of Cholinesterases

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This review is devoted to the application of various types of ion-selective electrodes in the study of the activities and catalytic properties of cholinesterases and also in chemical analysis with participation of these enzymes. Studies using a membrane "substrate" electrode, a glass pH electrode, an oxidation-reduction platinum electrode, and a polycrystalline electrode based on silver sulphide are considered. Investigations using enzyme electrodes based on immobilised cholinesterases are also described. The advantages and disadvantages of individual methods employing different types of ion-selective electrodes are discussed.
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I. INTRODUCTION

The special attention devoted to the study of cholinesterases (CE) can be explained not only by the physiological importance of this enzyme, which plays a significant role in the transmission of the nerve pulse, but also by its considerable practical importance in medicine, toxicology, pharmacology, specific syntheses of certain pesticides, and analytical chemistry. Certain diseases are diagnosed on the basis of the level of activity of this enzyme and the degrees of poisoning by certain organophosphorus compounds and carbamates are determined. Cholinesterase is the target of the action of certain insectoacaricides and specific synthesis of the latter therefore requires the knowledge of the specific features of the properties of cholinesterases of different animals. In analytical chemistry enzymatic analysis of organophosphorus compounds and carbamates based on cholinesterase activity is unique in its sensitivity and selectivity.

Cholinesterase is a highly specific catalyst of the hydrolysis of choline esters and their thio-derivatives and of the hydrolysis of certain aromatic and aliphatic carboxylate esters[1]. Cholinesterases which hydrolyse acetylcholine at the highest rate have been called true cholinesterases or acetylcholinesterases (ACE) (E.C.3.1.1.7), while cholinesterases which hydrolyse other choline esters (butyrylcholine, propionylcholine, benzoylcholine, etc.) at the highest rate are called pseudocholinesterases or cholinesterases (E.C.3.1.1.8). The natural substrate of cholinesterases is acetylcholine, in the enzymatic hydrolysis of which the products are an alcohol (choline) and acetic acid:

$$[CH_3CO{-}O{-}CH_2{-}CH_2{-}N(CH_3)_3]^+ + H_2O \overset{CE}{\rightleftarrows} CH_3COOH + [HO{-}CH_2{-}CH_2{-}N(CH_3)_3]^+.$$

All studies on cholinesterases associated with their catalytic properties are bound to include the determination of the enzyme activity or of the rate of enzymatic hydrolysis. Ion-selective electrodes have found extensive applications, among other metals, in the solution of these problems[2,3]. The high selectivity and fairly high sensitivity of the method and the possibility of automation combined with a comparative simplicity of the analysis and of the relevant apparatus have made ionometry an extremely attractive and sometimes irreplaceable procedure for the investigation of the properties and activities of cholinesterases as well as the analysis of their substrates, inhibitors, and activators[4,5].

Ion-selective electrodes sensitive either to the substrate, for example, to choline esters, or to the hydrolysis products (the alcohol or acid) can be used in studies on cholinesterases.

II. THE "SUBSTRATE" ELECTRODE

Only one ion-selective membrane electrode sensitive to choline esters, which has come to be called the substrate electrode, is used nowadays[6]. The electrode is based on a liquid membrane [5% solution of acetylcholine tetra-(*p*-chlorophenyl)borate in *o*-nitroxylene] and is the first ion-selective electrode sensitive to organic cations. This electrode was first proposed by Baum for the investigation of choline esters and was subsequently used by the same worker to determine the cholinesterase activities[7] and kinetic characteristics of ACE and CE [8,9] as well as their substrate specificities[9].

The study of the selectivity of this electrode with respect to different choline esters[9] showed that it increases from choline to benzoylcholine by a factor of 10^5 in the following sequence: choline < acetylcholine < propionylcholine and acetylmethylcholine < butyrylcholine < valerylcholine < benzoylcholine. The sensitivity of the electrode to alkali metal ions[7] proved to be very low compared with acetylcholine: thus the sensitivity to potassium and ammonium ions is smaller by a factor of 10^3 and that with respect to sodium ions by a factor as large as 10^4. The kinetic characteristics of the enzymatic hydrolysis (the Michaelis constant and maximum velocity) determined with the aid of the substrate electrode for the ACE of the electric organ of the eel and the CE of horse blood serum agree satisfactorily with the available literature values of these quantities[10]. Baum and Ward[9] also used the electrode successfully for the analysis of biological media—for the determination of the cholinesterase activity in blood.

A disadvantage of the "substrate electrode" method in kinetic research is the decrease of its sensitivity with increase of the concentration of choline esters, which virtually restricts the region of its applicability to a substrate concentration limit of a little more than 10^{-3} M, because the optimum version of the enzymatic hydrolysis and the determination of its rate from the change in the substrate concentration involves conditions in which 5% hydrolysis takes place during a time interval suitable for the experiment;

this cannot be expected for a substrate concentration greater than 10^{-3} M. The substrate electrode apparently cannot be used in the study of the action of irreversible inhibitors in inhibitor analysis either, because a large substrate concentration (approximately 10^{-2} M) is required to suppress the effect of the inhibitor[11-13]. Furthermore, one should note that a large group of reversible and some irreversible inhibitors of cholinesterases contain in their structure a quaternary nitrogen group[1] and can therefore have an appreciable influence on the readings of the substrate electrode.

III. THE USE OF THE GLASS pH ELECTRODE

Historically the first ion-selective electrode used to determine the activity of cholinesterases was the glass pH electrode[14,15]. A method based on measurements of the rate of the change of pH of the reaction medium owing to the accumulation of acetic acid in the latter during the enzymatic hydrolysis of acetylcholine in an unbuffered or weakly buffered medium has been proposed[16]. The author[16] used this method to investigate the cholinesterase activity of erythrocytes and plasma. It has been called the "ΔpH" method.

The exceptional simplicity of the "ΔpH" method and its high sensitivity attracted the attention of many investigators, who modified it by introducing a number of improvements[4,17-35]. The "ΔpH" method has been applied in clinical studies on cholinesterases[16,20-22,24,25,28], in toxicology[18,19,23,26], in pharmacology[26], in the study of inhibitors and insecticides[17,18,23], and in analytical chemistry for rapid mass analyses[5,36] (up to 50 analyses per hour). It is noteworthy that the cholinesterase enzyme electrodes available at present[5,37-40], which have been described above, are also based on the "ΔpH" method.

However, together with many useful qualities, the "ΔpH" method also suffers from a series of disadvantages. Firstly, the method requires additional calibration in order to obtain the absolute activities of cholinesterases[31-34]. Secondly, the sensitivity of the method depends markedly on the buffer capacity of the system, so that it can be used only for unbuffered and weakly buffered media. Thirdly, considerable errors are associated with the influence of atmospheric CO_2, which necessitates an effective protection from CO_2.

Some of these disadvantages can be avoided in the method of potentiometric titration at constant pH, where the acid accumulated during hydrolysis is titrated with alkali[14,15,41-49]. The titration can be carried out automatically[50-52].

In Yakovlev's modification[49] involving the use of a capillary burette the potentiometric titration method proved to be very convenient for kinetic research; it is difficult to point to a field of study of cholinesterases where it has not been used. Grigor'eva[53] proposed yet another improvement to the method—additional titration with the substrate simultaneously with the titration of the liberated acid with alkali, which made it possible to carry out studies at very low constant substrate concentrations (1×10^{-5} M). Such low substrate concentration can be used only in the substrate electrode method.

The potentiometric titration method has become a kind of standard for tests on other new methods for the investigation of the activity of cholinesterases. However, one should note the limitations of this method: its application is interfered with by atmospheric CO_2 and a high buffer capacity of the medium; comparatively large volumes of solutions of the reactants are required, since a decrease in the volume of the reaction medium entails an appreciable decrease in the accuracy of the method.

IV. OTHER ION-SELECTIVE ELECTRODES

As already mentioned, the hydrogen ion is the first product of cholinesterase hydrolysis which became the object of ionometric measurements. At the present time ionometry has advanced so much both qualitatively and quantitatively that it has become possible to use also other ions produced by the enzymatic hydrolysis in the study of cholinesterases. Ion-selective electrodes based on perfluoroacyl groups, sensitive to acetate, propionate, and butyrate ions, have been developed in the Ionometric Laboratory of Leningrad State University[54] and have been used to determine the activities of cholinesterases in the Laboratory of the Chemistry of Biologically Active Substances of the Institute of Evolutionary Physiology and Biochemistry. Experiments showed that the activity of cholinesterases can be determined in this way; however, the electrodes investigated proved to be insufficiently selective with respect to carboxylate anions in the presence of chloride and bromide anions in 100–1000-fold excess compared with the pyranil determined. The interfering ions cannot be eliminated or their elimination is fairly difficult. The successful application of this method therefore requires other anion-sensitive ion-selective electrodes with a high selectivity with respect to the test anion in the presence of Br^- and Cl^-.

The activity of cholinesterases can be studied in relation to another hydrolysis product—the alcohol, for example, choline, which contains a quaternary nitrogen cationic group. The choline electrode exists, but so far there has been no information about its applications in the analysis of cholinesterase hydrolysis.

Thiocholine has been more convenient for the application of ionometry. The point is that, owing to its SH group, thiocholine is a comparatively powerful reductant, being oxidised by many oxidants, including atmospheric oxygen, to the disulphide (the oxidation-reduction potential of this system is approximately 300 mV), which permits the use of oxidation-reduction electrodes for the determination of thiocholine. Apart from potentiometric methods amperometric and polarographic methods have been used for the determination of thiocholine[3,56]. This review deals only with potentiometric methods.

In 1962 Guilbault et al.[57] used platinum electrodes to determine the activity of cholinesterases with respect to thiol ethers on the basis of the amount of thiocholine. Two platinum electrodes and a reference electrode (a silver|silver chloride electrode) were placed in a buffer solution of the enzyme to which a solution of potassium iodide was added. A small constant current (5–25 μA) was passed through the solution; free iodine was then evolved at the anode in a small and constant amount. The platinum anode and the reference electrode were connected to a potentiometer. When a current was passed, a constant potential difference (approximately 400 mV) arose at the anode. After the addition of the thiocholine ether as the substrate to the reaction mixture, enzymatic hydrolysis began, which resulted in the appearance in the system of thiocholine, a more active reductant than I^-, which caused the depolarisation of the electrode and reduced the potential difference. The rate of decrease of potential in the same range, for example, between 300 and 100 mV, then proved to be directly proportional to the activity of cholinesterase and the final potential depended on the concentration of the thiocholine ether[4,58]. The method is satisfactorily sensitive and highly accurate (to within approximately 1.5%); it is possible to automate the analysis[59]. The inventors of the method have used it successfully to analyse organophosphorus compounds and carbamates[60]. It is noteworthy that the involvement of

platinum electrodes made it necessary to use the method with caution in the study of media with a large amount of protein, since platinum can be "poisoned" under these conditions.

In order to determine the amount of thiol obtained in the hydrolysis of thiocholine esters, an HS-sensitive ion-selective electrode with a polycrystalline membrane based on Ag_2S has also been used[5,61,62]. The method exhibits a high sensitivity combined with an adequate accuracy. However, the readings of the HS^- electrode are unstable in time, probably owing to the erosion of the surface, which appreciably restricts its use for the determination of the activity of cholinesterases[5]. More reliable results are obtained by potentiometric titration with a silver electrode[63].

V. ENZYME ELECTRODES USING CHOLINESTERASES

In the application of cholinesterases for analytical purposes, the device developed in Ref.64, which combines the immobilised enzyme with the corresponding ion-selective electrode, is convenient; it has been called the enzyme electrode. Enzyme electrodes exhibit a high selectivity and permit the analysis not only of ions, but also of non-ionic organic substances[65].

In Guilbault's review[5], devoted to the use of enzyme electrodes in analytical chemistry, a cholinesterase enzyme electrode is described, where cholinesterase solutions are immobilised on the surface of a glass pH electrode with a thin membrane†. Apart from the CE solution, Crochet and Montalvo[37] immobilised in this way a substrate solution, which enabled them not only to determine the concentration of inhibitors in the reaction medium, but also to estimate the activity of CE in biological samples. However, the method has not been used widely in practical applications owing to the technical difficulty of the preparation of the electrode and its short lifetime, which is largely due to the method of immobilisation of the enzyme.

The method of covalent binding of the enzyme to an inert protein has a considerable advantage in this respect. This method of immobilisation has been developed for acetylcholinesterase by a group of French investigators[66], which enabled them to prepare an acetylcholinesterase electrode[38,39] for the determination of the concentrations of acetylcholine and cholinesterase inhibitors—proserine and eserine. After 2 months, the activity of the immobilised enzyme decreased by 90%, while the sensitivity of the electrode to acetylcholine was 1×10^{-4} M. The dependence of the electrode potential on the logarithm of the acetylcholine (AC) concentration in the range 10^{-4}–10^{-2} M at pH 8.5 remained linear ($\Delta E/\lg [AX] \cong 35$ mV). With increase of acetylcholine concentration above 10^{-2} M, the electrode potential remained almost unchanged. Thus the acetylcholinesterase enzyme electrode for the measurement of the acetylcholine concentration is very inferior to the substrate electrode described above. However, in the measurement of the concentrations of certain ACE inhibitors, for example nicotine and particularly proserine (neostigmine) and eserine (physostigmine), the enzyme electrode proved to be extremely sensitive. Thus a linear dependence of this potential on the logarithm of the inhibitor concentration was observed up to 5×10^{-8} M in eserine solutions and up to 5×10^{-7} M in proserine solutions, which makes it possible to regard the enzyme electrode method as extremely promising for analysis.

VI. CONCLUSION

Comparison of the possibilities of a wide variety of methods—"ΔpH", potentiometric titration with a pH electrode, the substrate electrode, and Guilbault's oxidation-reduction method for thiocholine esters (see Table)—shows that, despite the large number of methods (including their modifications), none is universal. In each specific study it is

Brief characteristics of the principal potentiometric methods for the determination of the activity of cholinesterases (under optimum conditions).

Method	Ion-selective electrode	Ion being determined	Sensitivity of method			Accuracy of method	Limitations of method
			w.r.t. enzyme activity, mg^{-1}	w.r.t. substrate concn., M	w.r.t. concn. of hydrolysis product		
"ΔpH"	pH electrode (glass)	hydrogen ion	0.02	$(1-2) \times 10^{-4}$	1×10^{-5}	~10%	unbuffered and weakly buffered media; the presence of CO_2 interferes; special calibration is required
Potentiometric titration with alkali at constant pH	pH electrode (glass)	hydrogen ion	0.01–0.02	1×10^{-5}	1×10^{-5}	~4%	weakly buffered medium; the presence of CO_2 interferes; a large volume of reaction mixture (~10 ml) is required
Substrate electrode method	membrane ion-selective electrode sensitive to choline esters	organic cations: AC, PrC, BuC, and other choline esters	0.01–0.02	1×10^{-5}	—	~8%	high substrate concentrations ($> 10^{-3}$ M) cannot be used; the presence of other quaternary hydrogen compounds interferes
Oxidation-reduction potentiometry	oxidation-reduction electrodes	thiocholine ion	0.005	1×10^{-4}	1×10^{-5}	~1.5%	a protein medium interferes; additional apparatus for electrolysis is needed; the method requires calibration; only thiocholine esters can be used

†This electrode was made by Crochet and Montalvo in 1973.[37]

necessary to select carefully a suitable method which nevertheless introduces errors into the results, frequently exceeding the random errors of the measurements.

All this makes it necessary to seek and design new ion-selective electrodes whose application would improve the method for the investigation of cholinesterases. From our point of view, such research should be carried out by designing new, more sensitive "substrate" electrodes, anion-sensitive electrodes with a response to carboxylate ions and a high selectivity in the presence of halide ions, and a choline electrode with a high selectivity in the presence of choline esters.

A second approach involves the creation of new enzyme electrodes based on cholinesterase preparations. For its successful development, it is necessary to manufacture industrially highly active and stabilised cholinesterase preparations and to develop new methods for their immobilisation.

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