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BIOELECTRIC METHOD FOR THE DETECTION OF ANTICHOLINESTER-ASES

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Fig. 1

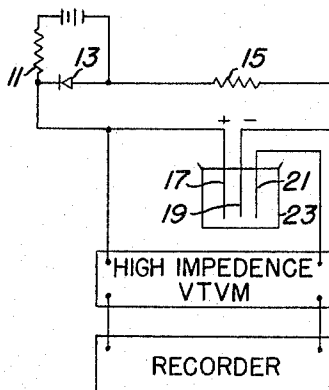


Fig. 2

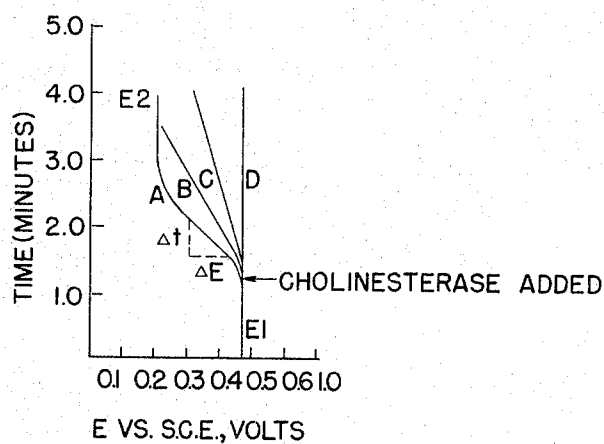
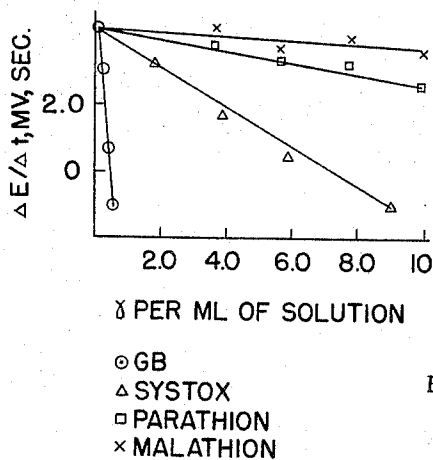


Fig. 3



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BIOELECTRIC METHOD FOR THE DETECTION OF ANTICHOLINESTERASES

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10 Claims. (Cl. 204—1)

The invention described herein may be manufactured and used by or for the Government of the United States of America for governmental purposes without the payment to us of any royalty thereon.

This invention relates to a new method for the detection of anticholinesterases. More specifically the present invention provides for the electrical monitoring of cholinesterase catalyzed reactions.

One of the advantages of the present invention is that it provides an accurate laboratory method for the remote determination of micro amounts of a highly toxic agent in a manner similar to the method set forth in the patent issued to Northrop 2,757,132.

Some of the organophosphorous compounds are highly toxic anticholinesterase agents. One of the more important enzymes in the higher animals is cholinesterase which is vital for the transmission of nerve impulses for muscle control. Minute amounts of anticholinesterase agents such as the G agents, which are disclosed in Patent 2,926,072, inactivate this enzyme and cause paralysis of the animals' muscular system and eventual death from failure of the respiratory system.

It is an object of this invention to detect amounts of these organo-phosphorous anticholinesterase agents in the order of millionths of a gram per milliliter of solution. It is a further object of this invention to detect the above minute amounts of these anticholinesterase agents with an accuracy of about $\pm 1\%$.

It has been proposed (U.S. Patent 3,049,411) to detect these agents by means of the reaction of cholinesterase with colorless acetates of indophenols. In the absence of an agent, the indophenolester is hydrolyzed to a highly colored compound. The present method is a distinct improvement over this patented method since it gives a quantitative indication of the presence of such agents and is not interfered with by extraneous colored solutions.

The above objects are achieved in this invention by applying a direct current across two platinum electrodes immersed in a buffered approximately neutral aqueous solution of the anticholinesterase compound and a thiocholine ester which also has a half cell or a reference electrode therein. The potential between the positive electrode and the reference electrode is then recorded as a function of time. Cholinesterase is then added to the solution and the time of addition is noted. There will be a change in the recorded potential as a function of time. This potential change per unit of time is the rate of depolarization of the positive electrode caused by the action of the cholinesterase on the substrate. This change in potential per unit of time or voltage-time is linearly related to the concentration of the enzyme. In the presence of anticholinesterase the activity of the enzyme is decreased as a function of inhibitor concentration. It has been found that the ratio is inversely proportional to the concentration of the inhibitor. Thus, a relatively large ratio indicates a relatively small amount of inhibitor present in the solution.

The thiocholine esters which can be used in this process are the acetyl, propionyl, and butyryl thiocholine iodides or the lower alkanoyl esters of ethanthiol trimethyl ammonium iodide. The pH of the solution in the concentration cell must be approximately neutral, i.e., in the

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range of pH from 6-8 with the preferred pH being 7.4.

In the drawing, FIG. 1 illustrates the electrical circuit with the vacuum tube volt meter and the recorder connected to the solution.

FIG. 2 shows the voltage-time curves for the enzymatic hydrolysis of butyrylthiocholine iodide by cholinesterase inhibited by; in case A, no agent; in case B, 0.36 $\mu\text{g.}$ per ml.; in case C, 0.50 $\mu\text{g.}$ per ml.; in case D, 1.0 $\mu\text{g.}$ per ml. of isopropyl methyl phosphonofluoridate (hereinafter designated as GB), respectively.

FIG. 3 shows the calibration plots of $\Delta E/\Delta t$ vs. the agent concentration in $\mu\text{g./ml.}$ for four typical anti-cholinesterase agents.

In FIG. 1, the apparatus used to apply a constant low amperage current and to measure the rates of depolarization is shown. A 135-volt battery is applied across two platinum thimble electrodes, 17 and 19 through a resistance of a 270-kilohm resistor, 11, and a 2-megohm resistor, 15. The circuit is stabilized by means of a Zener diode, 13. A reference half cell such as a saturated calomel electrode is shown at 21. The solution is contained in a concentration cell, 23, which is constantly stirred and maintained at a constant temperature by means of a standard water jacket or by location in a constant temperature chamber.

Example 1

Twenty-five ml. of a 1×10^{-3} M butyrylthiocholine iodide in 0.1 M aqueous tris(hydroxymethyl)amino-methane solution (pH 7.40) is placed in a 50-ml. beaker, and 1.0 ml. of an aqueous GB sample is added. This sample can contain 1 to 15 $\mu\text{g.}$ of GB or the agent which is to be analyzed. The resulting solution is magnetically stirred at a temperature of 25°C. , the platinum electrodes and the reference electrode are immersed into the solution and a current of 25 $\mu\text{a.}$ is applied across the two platinum electrodes by means of the circuit shown in FIG. 1. The recorder is switched on, and the resulting potential of the platinum anode vs. the reference electrode is automatically recorded. At zero time, 1.0 ml. of a 1.0 mg. per ml. cholinesterase solution is added to effect the enzymatic hydrolysis of the substrate.

Under operating conditions without the addition of GB, addition of cholinesterase to the magnetically stirred solution of the thiocholine ester caused a rapid hydrolysis of the substrate to thiocholine and butyric acid. The thiocholine produced causes a depolarization of the electrodes and a reduction in the potential of the system. This is illustrated by the aforementioned curves of FIG. 2. By means of predetermined calibration plots of the type set forth in FIG. 3 one can obtain the amount of agent present in the original sample. In a manner similar to Blaedel and Hicks (Analytical Chemistry, vol. 34, No. 3, March 1962, pages 388-394), one can calibrate the recorder to give a direct readout in micrograms of the agent. The temperature must be closely controlled to give the optimum results as far as accuracy is concerned.

We have found that the above procedure works well with GB and Systox (O,O-diethyl-O-[2-(ethylthio)-ethyl] phosphorothionate). However, for compounds such as parathion (diethyl-p-nitrophenyl thiophosphate) and malathion (S-[1,2-dicarbethoxyethyl]-O,O - dimethyl-dithiophosphate), we have found that these compounds must be preincubated with the enzyme for about ten minutes to give sensitive and accurate results. Likewise, with GB or Systox a short incubating period of 3-5 minutes made the process more sensitive to smaller amounts of these agents.

We have also found that with parathion and malathion and other water insoluble agents a cosolvent had to be used to get these agents into the solution. We have found that an aliphatic oxygenated solvent such as glycerol, ethylene glycol, diethylene glycol, diethylene glycol mono-

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ethyl ether, ethylene glycol monomethyl ether were excellent cosolvents to use in our process. Best results were obtained with the use of ethylene glycol monomethyl ether. It was found that up to about 1% of the aliphatic oxygenated solvent could be tolerated in the final reaction mixture without giving erratic results.

Our method can be applied to other agents or inhibitors such as GA, GD, paraoxon, malaoxon, and DFP with equally good results.

We claim:

1. A method of quantitatively detecting the presence of organophosphorus compounds which comprises:

(a) applying a low direct current across a negative and a positive electrode immersed in a stirred buffered aqueous solution of said compound and a lower alkanoyl thiocholine ester having a reference electrode therein,

(b) recording the potential between said positive electrode and said reference electrode,

(c) adding cholinesterase to said solution,

(d) recording the ratio of the change in potential between said positive electrode and said reference electrode with respect to the change in time from the addition of said cholinesterase, said ratio being inversely proportional to the concentration of said compound in said buffered solution.

2. A method of quantitatively detecting the presence of organophosphorus compounds which comprises:

(a) applying a weak direct current across a negative and a positive electrode immersed in a neutral aqueous solution of said compound and a lower alkanoyl thiocholine ester which has a reference electrode therein,

(b) recording the potential between said positive electrode and said reference electrode,

(c) adding cholinesterase to said solution,

(d) recording the ratio of the change in potential between said positive electrode and said reference electrode with respect to the change in time from the addition of said cholinesterase, said ratio being inversely proportional to the concentration of said compound in said neutral solution.

3. A method of quantitatively detecting the presence of isopropyl methyl phosphonofluoridate which comprises:

(a) applying a direct current of low amperage across a negative and a positive electrode immersed in a stirred buffered approximately neutral aqueous solution of said phosphonofluoridate and a lower alkanoyl thiocholine ester having a reference electrode therein,

(b) recording the potential between the positive electrode and said reference electrode,

(c) adding cholinesterase to said solution,

(d) recording the ratio of the change in potential between said positive electrode and said reference electrode with respect to the change in time from the addition of said cholinesterase, said ratio being inversely proportional to the concentration of said phosphonofluoridate in said buffered solution.

4. A method of quantitatively detecting the presence of water soluble organophosphorus compounds which comprises:

(a) applying a weak direct current across a negative and a positive electrode immersed in a stirred aqueous

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solution containing said compound, tris(hydroxymethyl)amino-methane, and butyrylthiocholine iodide said solution being adjusted to a pH range of 7.0 to 8.0 and having a reference electrode therein,

(b) recording the potential between said positive electrode and said reference electrode.

(c) adding cholinesterase to said solution,

(d) recording the ratio of the change in potential between said positive electrode and said reference electrode with respect to the change in time from the addition of said cholinesterase, said ratio being inversely proportional to the concentration of said organophosphorus compound in said solution.

5. A method as set forth in claim 4 in which the organophosphorus compound is isopropyl methyl phosphonofluoridate.

6. A method of quantitatively detecting the presence of water insoluble organophosphorus compounds which comprises:

(a) applying a weak direct current across a negative and a positive electrode immersed in a stirred aqueous solution containing said compound, tris(hydroxymethyl)aminomethane, butyrylthiocholine iodide, and up to about 1% of the total solution of a solvent selected from the group consisting of glycerol, ethylene glycol, diethylene glycol, diethylene glycol monomethyl ether, and ethylene glycol monomethyl ether, said solution being adjusted to a pH range of 7.0 to 8.0 and having a reference electrode therein,

(b) recording the potential between said positive electrode and said reference electrode,

(c) adding cholinesterase to said solution,

(d) recording the ratio of the change in potential between said positive electrode and said reference electrode with respect to the change in time from the addition of said cholinesterase, said ratio being inversely proportional to the concentration of said organophosphorus compound in said solution.

7. A method as set forth in claim 6 in which the solvent is ethylene glycol monomethyl ether.

8. A method as set forth in claim 7 in which the organophosphorus compound is O,O-diethyl O-2-(ethylthio)-ethyl phosphorothionate.

9. A method as set forth in claim 7 in which the organophosphorus compound is diethyl-p-nitrophenyl thiophosphate.

10. A method as set forth in claim 7 in which the organophosphorus compound is S-(1,2-dicarbethoxyethyl)-O,O-dimethyldithiophosphate.

References Cited by the Examiner

UNITED STATES PATENTS

2,757,132	7/1956	Northrop	204—195
2,926,072	2/1960	Kramer et al.	23—232
3,049,411	8/1962	Gelman et al.	23—232

OTHER REFERENCES

Blaedel et al.: "Analytical Chemistry," vol. 34, No. 3, March 1962, pp. 388—394.

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