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LETTER DATED 16 MAY 1991 FROM THE DEPUTY PERMANENT REPRESENTATIVE OF AUSTRIA TO THE SECRETARY-GENERAL OF THE CONFERENCE ON DISARMAMENT TRANSMITTING A STUDY ENTITLED "DETECTION OF INHIBITORS OF THE ENZYME ACETYLOCHOLINE ESTERASE OVER LONG DISTANCES USING OPTICAL FIBERS"

I have the honour to forward to you a copy of an Austrian study entitled "Detection of inhibitors of the enzyme acetylocholine esterase over long distances using optical fibers" and I would ask you to be kind enough to circulate it as an official document of the Ad Hoc Committee on Chemical Weapons as well as of the Conference on Disarmament.

(Signed) Christian Strohal
Deputy Permanent Representative
to the Conference on Disarmament

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Project

Detection of inhibitors of the enzyme acetylcholine esterase over long distances using optical fibers

AUSTRIAN SCIENTIFIC COUNCIL FOR THE IMPLEMENTATION OF THE CHEMICAL WEAPONS CONVENTION

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Univ. Prof. Dr. Otto S. Wolfbeis
Mag. Dr. Wolfgang Trettnak
Mag. Dr. Michael Hofer
Dipl. Ing. Franz Reininger
Mag. Ernst Zinterl

Abstract

Many pesticides (such as organophosphates or carbamates) and most nerve agents used in chemical warfare (such as Tabun, Sarin or VX) are potent inhibitors of a group of enzymes present in humans and animals, called choline esterases. The activity of one of these esterases, the acetylcholine esterase (AChE), is essential for the function of the nerve system. An inhibition of the AChE in humans leads to sweating, headache and running nose in the case of a minor contamination. If the AChE is completely inhibited, cramps, paralyzation of the breath center and finally the death are the result.

Pesticides are commonly found in ground water and drinking water due to their extensive use in agriculture as crop protection agents. The production and the transport of these substances represents another area of possible contamination of humans and of the environment (i.e. Cordoba, Mexico, May 1991). As recent political developments have shown, the use of chemical weapons still threatens millions of people. The rapid and accurate detection of inhibitors of the acetylcholine esterase therefore represents an important preventive procedure for the protection of humans, animals and environment.

We have developed a new method for on-line detection of inhibitors of the AChE in a continuous flow system. The presence of inhibitors is detected optically through fibers. Therefore, the sensing part of the apparatus can be as far as 1000 meters and more away from the detecting part. The detection principle combines low detection limits (for example 10 nM of the potent reversible inhibitor BW 284 C51) with remote sensing, cheap and robust solid state technology (light emitting diodes as light sources and photodiodes as light detectors) and ease of operation. The detection limits for the presence of inhibitors of the AChE can be enhanced by one to two orders of magnitude. This can be achieved through an optimization of the electronical, chemical and optical components of the system without major increase of the costs of the system.

Otto S. Wolfbeis, Wolfgang Trettnak, Michael Hofer, Franz Reininger, Ernst Zinterl

THE DETECTION OF INHIBITORS OF THE ENZYME ACETYLCHOLINE ESTERASE OVER LONG DISTANCES USING OPTICAL FIBERS

1. The detection of inhibitors of the enzyme acetylcholine esterase: concern and pertinence [1-10]

Acetylcholine is the most important chemical transmitter in the nerve system of humans and animals (Fig.1). If a sense-organ is stimulated by an external event, the excitation is transformed into bioelectrical impulses which are transmitted through the nerve fibers to the central nerve system and/or the brain.The nerve fibers consist of many nerve cells which are connected to each other by a slit, called synapse. The electrical information is transmitted chemically across the synapse between two nerve cells. The transmitter substance in this chemical reaction is the acetylcholine. It is released at one side of the synapse through the excitation arising from the electrical impulse of one nerve cell. Acetylcholine then diffuses across the slit to the other side of the synapse and excites the next nerve cell. In fact, the transmission of the stimulation information of the sensing organ can be viewed as succeeding reaction of many electrical and chemical transmission steps.

Fig. 1

After excitation of the nerve cell, the acetylcholine molecule has to be destroyed in order not to excite continuously the nerve cell. This deactivation is performed by the enzyme acetylcholine esterase which hydrolyzes the acetylcholine into choline and acetic acid. An inhibition of the enzyme acetylcholine esterase results in an incomplete deactivation of the transmitter acetylcholine. The consequences in humans are sweating, headache and running nose in the case of a minor inhibition. If the acetylcholine esterase is completely inhibited, cramps, paralyzation of the breath center and finally the death are the result. The inhibition of the enzyme may be reversible or irreversible, depending on the desired area of application. Since many inhibitors of the acetylcholine esterase are commonly used in civil as well as military tasks, the importance of a detection method for these substances becomes obvious.

Several classes of substances are potent inhibitors of the enzyme acetylcholine esterase: Organophosphates, carbamates, quarternary ammonium ions and methanesulfonates. Organophosphorus inhibitors of the acetylcholine esterase have found application in two main areas: as pestizides in agriculture (i.e. E 605) and as chemical weapons (i.e. Sarin, Tabun). Chemical warfare agents and pesticides cannot be separated strictly since some organo-phosphorus compounds have served many years both purposes. The main difference between the pesticides and the chemical weapons is the choice of the substitutents at the phosphorus atom during syntheses. For example, fluoride has been used as a substituent in chemical weapons to enhance the speed of inhibition of acetylcholine esterase. Carbamates are used extensively in the crop protection. Quarternary ammonium ions and carbamates both have served in medicine as parasympathomimetic agents.

2. The determination of inhibitors of the enzyme acetylcholine esterase

The most widely-used methods for the determination of organophosphorus and carbamate pesticides are high-pressure liquid chromatographie (HPLC), gas chromatographie (GC) and mass spectroscopy (MS) [11]. All of these methods require a tedious sample pretreatment, sophisticated instrumentation and are expensive.

To detect inhibitors of the enzyme acetylcholine esterase (AChE) it is advantageous to use the enzyme itself. For this purpose the activity of the enzyme is measured in the presence of an inhibitor after an incubation time of typically 30 minutes. Many methods have been described which differ in the way in which the activity of the enzyme is determined [11,12].

The natural substrate of the enzyme is acetylcholine which is cleaved by acetylcholine esterase to give acetate and choline (Equ. 1). In a radiometric assay acetylcholine was labelled with tritium (H^3) or with carbon C^{14} at the acetyl group. After reaction with the enzyme, the acetic acid produced was separated and its radioactivity was measured [11,12]. The problems of this method are the radioactive labelling of the acetylcholine, the radioactive waste, which has to be dispose and the radioactivity itself.

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{acetate} + \text{choline} \qquad \text{(Equ. 1)}$$

In electrochemical methods the production of acetic acid (Equ. 1) was followed with a pH electrode [12] or with a pH sensitive field effect transistor (ISFET) [13]. Very often, the reaction of acetylcholine esterase is coupled to a second enzymatic reaction. The enzyme choline oxidase (ChOD, EC 1.1.3.17) oxidizes choline and as products betaine and hydrogen peroxide are formed (Equ. 2). The hydrogen peroxide can be detected amperometrically with a platinum electrode [14,15].

$$\text{choline} + 2\,O_2 + H_2O \xrightarrow{\text{ChOD}} \text{betaine} + H_2O_2 \qquad \text{(Equ. 2)}$$

In an other method, acetylthiocholine iodide has been used instead of acetylcholine and the formation of thiocholine was monitored potentiometrically [12].

Synthetic enzyme substrates are very often used in optical methods for the determination of inhibitors of acetylcholine esterase. The substrates and products usually show different colours and the colour changes occuring during the enzymatic reaction can be detected photometrically. A typical example is the yellow substrate indophenyl acetate, which is hydrolyzed by cholinesterases producing an intensely blue product [11].

Fluorescent dyes have also been used in detecting inhibitors of acetylcholine esterase. Indoxylacetate or resorufinacetate show a strong fluorescence after hydrolysis by the enzyme whereas the substrates do not show fluorescence. Unfortunately the excitation wavelengths of most of these compounds are in the ultraviolet region of the light [12].

Other colorimetric methods are based on complex reactions to produce a dye whose absorption is measured. The method from Ellman is well known [12]: Acetylthiocholine is hydrolyzed by acetylcholine esterase and the thiocholine formed reacts with 5,5′-dithiobis-(2-nitrobenzoate) to give a yellow dye.

Immunological reactions have also been used for the determination of pesticides. In an immunoassay for paraoxon, the high selectivity and sensitivity of an antigen/antibody reaction was coupled with the specificity of an enzymatic reaction [11]. Although this method was highly sensitive for paraoxon, it could be used for the detection of this single pesticide only. In addition it was very expensive and time consuming.

In this report we present a novel optical method for the determination of inhibitors of acetyl- and butyrylcholine esterases. We use synthetic enzyme substrates which are yellow in aqueous solution and are converted into blue products by choline esterases. In

absence of an inhibitor, all substrate is hydrolyzed by the enzyme to give the blue product. In presence of an enzyme inhibitor the amount of blue dye formed is reduced. This causes a change in colour, which may serve as an alarm for the presence of enzyme inhibitors for example in a drinking water reservoir. As the enzyme substrate is the indicator itself, no additional chemical reactions (and therefore no additional reagents) are required. The enzymes are immobilized covalently on glass or polymer particles and can be used over several days continuously in an enzyme reactor.

The blue colour of the products allows the detection with the help of a yellow light emitting diode (LED) as the light source. Cheep LED´s with high light out-put are available for longer wavelengths of the visible spectrum only. The use of optical fibers makes tit possible to perform measurements over large distances and at inaccessible places. Additionally, in this work we use a complete new type of a photometer, which is equipped with inexpensive components like LED´s as light sources, photodiodes as detectors and plastic fibers. With this instrument, a simple and cheap instrumentation is possible, in contrast to the expensive commercial photometers.

If lasers are used as light sources in connection with optical glass fibers, measurements can be performed over distances of at least several hundred meters. The procedure was designed as an on-line method for continuous measurement. As all substances which inhibit the enzymes acetylcholine esterase and butyrylcholine esterase can be detected, a kind of a sum of parameters is measured. We assumed that it is of interest to detect any substance which is harmful to the human nerve system and therefore to the whole organism. We were not looking for a method allowing the identification or quantification of pesticides or nerve gases in drinking or ground water. Of course the method shows different sensitivities towards the various inhibitors due to the different inhibiting properties of the substances.

3. Experimental

3.1. Materials

The enzymes acetylcholine esterase (EC 3.1.1.7; type VI-s, from electric eel) with a specific activity of 200 U/mg and butyrylcholine esterase (EC 3.1.1.8; from horse serum) with a specific activities of 360 or 308 U/mg were obtained from Sigma Chemie GmbH (Deisenhofen, FRG).

The synthetic enzyme substrates were Substrate A (with an acetoxy group) and Substrate B (with a butyroxy group). These compounds are proprietary substances.

The enzyme inhibitors paraoxon (diethyl-p-nitrophenylphosphate, E 600; 95 % liquid), pyridostigmine bromide and 1,5-bis(allyldimethyl-ammoniumphenyl)-pentane-3-one dibromide (BW 284 C51) as well as the reactivating reagent pyridine-2-aldoxime methoiodide (2-PAM) were purchased from Sigma Chemie GmbH (Deisenhofen, FRG).

Controlled pore glass Aminopropyl-CPG-170-A (120 - 200 mesh; with 0.2 mmol amino groups/g) was from Fluka Chemie AG (Buchs, Switzerland). Polymer beads with oxiran groups on the surface (300 µmol/g; particle size 50 - 200 µm) were obtained from Riedel-de Haen AG (Seelze, FRG). Nylon nets (with pore sizes of 30 and 60 µm) for sealing the enzyme reactors were purchased from Reichelt Chemietechnik GmbH + Co (Heidelberg, FRG).

All other chemicals used were of analytical-reagent grade.

3.2. Immobilization of the enzymes

The enzymes acetylcholine esterase from electric eel and butyrylcholine esterase from horse serum were used because they are available with high specific activities - a fact that is essential for the immobilizazion of the enzymes. We tried to immobilize both enzymes on different materials, such as controlled pore glass (modified with aminopropyl groups) or polymer particles (with oxiran groups on the surface). The glass or polymer beads

with the immobilized enzyme were filled in enzyme reactors with a reactor length of 1 cm and a volume of approximately 70 µl. The enzyme reactors were sealed on both ends with nylon nets and stored in buffer solution at 4 °C in a refrigerator untill they were used.

3.2.1. Immobilization on aminopropyl-CPG with carbodiimide

3.1 mg of acetylcholine esterase (620 units) or 2.2 mg of butyrylcholine esterase (677 units) was dissolved in 3.0 ml of 0.1 M phosphate buffer pH 7.0. After the addition of 0.2 g of aminopropyl-CPG and 50 mg of N-ethyl-N´(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), the solution was stirred for one hour at room temperature and stored at 4 °C for 24 hours in a refrigerator. The CPG was washed with cold 0.1 M phosphate buffer pH 7.0 and stored in the same buffer at 4 °C.

3.2.2. Immobilization on aminopropyl-CPG with glutaraldehyde

This immobilization method was adapted from a published procedure [7]. One ml of a 2.5 % solution of glutaraldehyde in 0.1 M phosphate buffer pH 7.0 was added to 0.2 g of aminopropyl-CPG. Before and after addition of the CPG, nitrogen was bubbled through the glutaraldehyde solution. After standing for one hour, the CPG was washed with water. 3.47 mg of acetylcholine esterase (694 units) or 1.8 mg of butyrylcholine esterase (648 units) were dissolved in 3.0 ml of cold 0.1 M phosphate buffer pH 6.0. The preactivated CPG was added to the enzyme solution and left to stand in a refrigerator at 4 °C for 2.5 or for 24 hours, respectively. The CPG was washed with cold 0.1 M phosphate buffer pH 6.0 and stored in the same buffer at 4 °C.

3.2.3. Immobilization on polymer particles via oxiran groups

3.40 mg acetylcholine esterase (646 units) or 1.03 mg of butyrylcholine esterase (317 units) were dissolved in 3.0 ml of 1 M potassium phosphate buffer pH 7.5. Then 2.53 or 1.59 mg of VA-Epoxy Biosynth (polymer beads with oxiran groups on the surface) were added, respectively. After two days of storage in a refrigerator at 4 °C, the polymer beads were washed with 0.1 M phosphate buffer of pH 7.4 and stored in 0.1 M phosphate buffer pH 7.0 at 4 °C.

3.3. The enzyme substrates

The enzyme substrates (Substrate A and Substrate B) are both highly soluble in water and water/solvent mixtures. The substrate solutions show a yellow colour and the absorption maxima in destilled water are at about 390 nm, whereas the products of the enzymatic hydrolysis are blue with absorption maxima at about 580 nm (Fig. 2).

The substrates display a good stability in destilled water, whereas they are less stable in solvents like ethanol or dioxan. In addition, they are also not stable in aqueous buffered solutions. Only in mixtures of water and cellosolve (2-ethoxy-ethanol) the stability of the substrate was highly improved. Unfortunately cellosolve led to a rapid decrease in enzyme activity which may be due to inhibition or denaturation of the enzyme. Therefore only substrates dissolved in distilled water were used. Figs. 3 and 4 show the spontaneous hydrolysis of the substrates in distilled water as a function of time and temperature. The solid substrates, which are orange-red cristalls, were stored below 0 °C under exclusion of moisture. No changes were observed over a period of several weeks.

Figs. 2, 3 & 4

3.4. The inhibitors

Numerous inhibitors of the enzymes acetyl- and butyrylcholine esterase are known [8]. We chose three representative examples of the most important substance groups: an organophosporus compound (paraoxon), a carbamate (pyridostigmine bromide) and a quarternized ammonium ion (BW 284 C51).

From paraoxon (liquid, 95 %) a 0.16 M stock solution was prepared in acetone. Aqueous 160 µM stock solutions were obtained by diluting this solution with destilled water. Pyridostigmine bromide and BW 284 C51 were cristalline and were dissolved in distilled water directly. The aqueous carbamate and paraoxon solutions were prepared fresh every day. For decontamination, the pesticide solutions and the contaminated materials were treated with 0.1 M solution of potassium hydroxide to hydrolyze paraoxon and pyridostigmine. BW 284 C51 was destroyed with the help of an alkaline solution of potassium permanganate.

3.4.1. Paraoxon (Diethyl-p-nitrophenylphosphate; E 600)

Paraoxon (Fig. 5a) is a well known example of the organophosphorus pesticides. The oral lethal dose LD50 is 1.8 mg/kg for rats and 5 mg/kg for men. Paraoxon is also a metabolite of the less toxic parathion (E 605) which has found wide application as pesticide. It is transformed into the highly toxic paraoxon in the body and the lethal dose is 300 - 400 mg for an adult man [2,4,17].

Paraoxon inhibits both, acetyl- and butyrylcholine esterase irreversibly by phosphorylation of the active site of the enzyme. Although the spontaneous regeneration of the enzyme is very slow (with half-life times of the phosphorylated enzyme in the order of days), it is possible to reactivate the enzyme with substances like pyridine-2-aldoxime-methoiodide (2-PAM, Pralidoxim). This reactivation is possible before the diethylphosphate group is partially hydrolyzed ("aging" of the phosphorylated enzyme) [1,3]. Since aging is very slow in the case of paraoxon as the inhibitor, the reactivation with 2-PAM occurs fast [11,13,14].

Fig. 5

3.4.2. Pyridostigmine bromide (3-Dimethylaminocarbonyloxy-N-methyl-pyridinium bromide; Mestinon)

Pyridostigmine bromide (Fig. 5b) was chosen, because it also inhibits both, acetyl- and butyrylcholine esterases [18]. The inhibition occurs through carbamylation of the enzyme, but the hydrolysis of the ester formed is fast when compared with the phosphorylated enzyme. The half-life times of carbamylated enzymes are in the order of 1 to several hours (depending on the type of enzyme) [1]. Pyridostigmine has been used as a so called parasympathomimetikum in medicine [2,3].

3.4.3. BW 284 C51 (1,5-bis(4-Allyldimethyl-ammoniumphenyl)-pentane-3-one dibromide)

The compound BW 284 C51 (Fig. 5) is a typical representative of inhibitors of the type of substituted ammonium ions. It is a potent reversible inhibitor of acetylcholine esterase and has a LD50 of 2.1 mg/kg for mice. It can bind to the anionic binding site of the enzyme and it has been used as a selective inhibitor for acetylcholine esterase in medical research [18].

3.5. Buffer solutions

Buffering of the substrate solutions in the enzyme reactor is essential because the enzyme activity and the enzyme inhibition are both pH dependent [6,17]. The addition of sodium

chloride and magnesium chloride may enhance the lifetime of the immobilized enzyme considerably. It was reported, that in the presence of 12 µM $MgCl_2$ and 45 mM NaCl the lifetime was three times higher than in absence of these salts [17]. Therefore all experiments were performed with buffers containing similar salt additions. Depending on the mixing ratio of buffer:substrate:inhibitor solutions we used 0.2 M sodiumphosphate buffer containing 180 mM NaCl and 48 µM $MgSO_4$ for a ratio of 1:1:2 and 0.1 M sodiumphosphate buffers containing 90 mM NaCl and 24 µM $MgSO_4$ for a ratio of 1:1:1. Buffer and substrate solutions had to be separated because the substrate is not stable in buffered solutions especially at pH > 7.

4. Measurements with an UV/VIS-spectrophotometer

The experimental set-up is shown in Fig. 6. The enzyme substrate solutions are mixed with buffer and inhibitor solution via a peristaltic pump and are pumped through an enzyme reactor. Here, the yellow substrate is hydrolyzed by the enzyme and the blue product is formed. In presence of an inhibitor the formation of this blue product is reduced because the enzyme is inhibited. This causes a change in absorbance at 580 nm which can be monitored with an UV/VIS-spectrophotometer.

Fig. 6

The spectrophotometer was a Perkin-Elmer Lamda 5 UV/VIS Spectrophotometer (Perkin-Elmer & Co GmbH, Überlingen, FRG). A flow-through cell with a light path of 1.5 mm from Hellma GmbH & Co (Müllheim, Baden, FRG) was used for the continuous flow measurements. For investigating the substrate stabilities 1x1-cm quartz cuvettes were used. The spectrophotometer allowed to measure absorption spectra in the flow-through cell which was advantageous in the beginning of the studies.

The peristaltic pump was a Gilson Minipuls 3 (Gilson Medical Electronics, Villiers-le-Bel, France) or a MV-CA4 from ISMATEC GmbH (Wertheim, Mondfeld, FRG).

The inhibitor solution represents a drinking or ground water reservoir, from which a sample is drawn continuously. In absence of an inhibitor, destilled water was used instead of the inhibitor solution. The mixing ratio was 1:1:1 for substrate:buffer:inhibitor at the beginning and was changed to 1:1:2 to increase the sensitivity of the method later on.

All measurements were performed at room temperature, i.e. 25 ± 1 °C.

4.1. Measurements with acetylcholine esterase immobilized on aminopropyl-CPG

Immobilization of acetylcholine esterase on aminopropyl-CPG with carbodiimide or glutaraldehyde yielded a material with high enzyme activity and good stability. After storage in a refrigerator (5 °C) for four months, no considerable loss in enzyme activity was observed. An enzyme reactor with acetylcholine esterase immobilized on aminopropyl-CPG with carbodiimide could be used for more than 50 hours (over a period of two weeks) in continous flow and was exposed to numerous inhibition tests with paraoxon, pyridostigmine or BW 284 C51. Enzyme reactors which were not in use, were stored at 5 °C in a refrigerator.

Fig. 7 shows the pH-dependence of the hydrolysis of Substrate A with a fresh prepared enzyme reactor. A broad pH-optimum from approximately pH 5.5 to 9.0 can be observed, which is due to a high enzyme activity in the enzyme column. A change in the flow-rate did not affect the results in the range of 0.5 to 1 ml/min.

Fig. 7

4.1.1. Inhibition with paraoxon

The inhibition of acetylcholine esterase with paraoxon is shown in Fig. 8. The changes in absorbance were measured after a time of 21 minutes. Paraoxon is an irreversible inhibitor of the enzyme. Therefore the concentration of the blue product decreases continuously with time since more enzyme gets inhibited. After the inhibition reaction, the reactivation was performed with 0.1 mM solution of 2-PAM and the enzyme activity was restored (Fig. 9). The detection limit was about 0.2 µM of paraoxon using this method.

Figs. 8 & 9

Investigation of the pH-dependence of the inhibition reaction showed an optimal pH-range of approximately 7.5 to 8.5 (Fig. 10). The effect of the flow-rate on the inhibition is shown in Fig. 11. The signal changes increased in the range of 0.4 to 2.0 ml/min.

Figs. 10 & 11

4.1.2. Inhibition with pyridostigmine bromide

The inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with pyridostigmine bromide is shown in Fig. 12. The changes in absorbance were measured after 21 minutes, although - in contrast to paraoxon inhibition - a steady-state response is established after some time. The limit of detection was about 1 µM of pyridostigmin. The regeneration times were 15 to 30 minutes and depended on the concentration of the inhibitor. Reactivation of the enzyme occured spontaneously and was not influenced by substances like 2-PAM.

Fig. 12

4.1.3. Inhibition with BW 284 C51

Fig. 13 depicts the inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with the reversible inhibitor BW 284 C51. A steady-state was observed after a time of about 5 to 15 minutes (Fig. 14). The regeneration times varied from 8 to 25 minutes (depending on the concentration of the inhibitor). The detection limit was approximately 10 nM of BW 284 C51.

Figs. 13 & 14

An increase in the flow-rate caused an increasing sensitivity (Fig. 15). The reason may be the high affinity of the enzyme to the inhibitor. Because of the two positive charges BW 284 C51 is more readily bound to the anionic binding site of the enzyme than the enzyme substrate.

Fig. 15

4.2. Measurements with acetylcholine esterase immobilized on VA-Epoxy-Biosynth

VA-Epoxy-Biosynth polymer beads with immobilized acetylcholine esterase also showed high enzyme activity and were suitable for inhibition tests. The inhibition of acetylcholine esterase with paraoxon is shown in Fig. 16. The signal changes were again measured after 21 minutes. The limit of detection was approximately 0.5 µM paraoxon and the reactivating times ranged from 10 to 20 minutes.

Fig. 16

4.3. Measurements with immobilized butyrylcholine esterase

The immobilization of butyrylcholine esterase was less successful than the immobilization of acetylcholine esterase. Enzyme reactors which were filled with butyrylcholine esterase immobilized on aminopropyl-CPG with carbodiimide showed almost no enzyme activity. Substrate B and Substrate A were not hydrolyzed. Immobilization of butyrylcholine esterase on aminopropyl-CPG with glutaraldehyde yielded better results. Fig. 17 shows the pH-dependence of the hydrolysis of Substrate B. A distinct pH-optimum of pH 7 can be observed. The signal was dependent on the flow-rate (Fig. 18) which indicates a low enzyme activity on the carrier material. At pH 7.5 and at a flow-rate of 1.8 ml/min only 50 % of the substrate were converted into the blue product. Enzyme reactors with butyrylcholine esterase immobilized on VA-Epoxy-Biosynth also showed low enzyme activities. At pH 7.5 and at a flow-rate of 1.8 ml/min only 25 % of the substrate were hydrolyzed.

Immobilized butyrylcholine esterase was inhibited by paraoxon or pyridostigmine, but almost no reactivation of the inhibited enzyme was observed. After inhibition with paraoxon it was, for example, not possible to reactivate the enzyme with 2-PAM. Only a small part of the enzyme activity was restored. Even inhibition with pyridostigmine was irreversible. Therefore it was not possible to determine detection limits or calibration graphs because each enzym reactor could be used for one inhibition test only before it had to be replaced. For that reason enzyme reactors with immobilized butyrylcholine esterase were not suitable for investigation and test purposes. Nevertheless, they could be used in a pesticide alarm system, where a single response is sufficient.

Figs. 17 &18

5. Measurements performed with a fiber optic photometer and a laser

Fig. 19 shows the experimental set-up for the measurements with the fiber optic photometer. The arrangement was the same as described in Chapter 4. Instead of the UV/VIS spectrophotometer, the 3-Lambda-Photometer from the Institute for Optical Sensors (Joanneum Research, Graz, Austria) was used. This photometer is equipped with solid-state components, such as light-emitting diodes (LED´s) as light sources and photodiodes as detectors. The photometer was connected to the flow-through cell with plastic fiber light guides. The flow-through cell was made of stainless steel plates with a teflon spacer in the centre. The light path through the cell was 2.5 mm long. In addition, the photometer signal was recorded with a Gould BS-273 three-channel recorder (Gould Bryans Instruments Ltd., Mitcham, Surrey). For all measurements, a yellow LED was used as the light source.

For measurements with the laser as the light source, a Helium-Neon-Laser (PMS Electro-Optics, Boulder, Colorado, USA) with an emission wavelength of 612 nm was used. The detector was a lightwave multimeter 8153A equipped with an optical head 81520A from Hewlett-Packard (Vienna, Austria). Plastic fibers with a thickness of 1000 µm and glass fibers with a thickness of 125 µm, as well as optical fiber couplers, were obtained from Hirschmann (Vienna, Austria).

Fig. 19

5.1. Measurements with the 3-Lambda-Photometer

For testing the 3-Lambda-Photometer the sample flow passed first the flow-through cell of the UV/VIS spectrophotometer and second the flow-through cell of the fiber-optic photometer in order to perform simultaneous measurements with two detectors. For most of the experiments, an overall length of 10 meters of plastic fibers was used. It was shown, that almost no differences could be observed between the results obtained with both photometers, although the fiber-optic photometer did not use a reference beam.

The inhibition of acetylcholine esterase (immobilized on polymer beads) with paraoxon is shown in Fig. 20. The results are identical with that from Fig. 16, which were obtained with the spectrophotometer. Only minor differences can be observed between the two calibration graphs.

Fig. 20

The inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with pyridostigmine is shown in Fig. 21. The calibration graph is almost identical with that from Fig. 12, which was measured simultaneously.

Fig. 21

A total of 90 meters of plastic fibers was used in the measurement shown in Fig. 22. The main difference between the measurements with 10 and 90 meters of plastic fibers was the smaller signal/noise ratio when 10 meters of plastic fibers were used. The limit of detection was approximately 30 nM for BW 284 C51. However, a length of 100 meters of plastic fibers appeared to be the upper limit when using the 3-Lambda-Photometer with a LED as the light source.

Fig. 22

5.2. Measurements with a laser

The inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with BW 284 C51 (Fig. 22) was repeated with a He-Ne-laser as the light source. The result (Fig. 23) is comparable to that obtained with the 3-Lambda-Photometer despite some differences in the central part of the calibration graph. 90 meters of plastic fibers were used again, but the signal/noise ratio was not as high as with the 3-Lambda-Photometer. This may be due to problems with coupling the light of the laser into the fiber and to laser instabilities. Little changes in the connection of fiber coupler and laser resulted in high signal fluctuations. The detection limit was approximately 50 nM for BW 284 C51.

Fig. 23

Nevertheless, these measurements demonstrate the usefulness of a laser as a light source. With the high output of a laser combined with the use of glass fibers it is possible to measure the signal changes which occure during the inhibition of choline esterases over large distances. A detection of inhibitors over a distance of about 100 meters is possible with the orange He-Ne-laser and with 1000-µm plastic fibers. With the same laser and 125-µm glass fibers measurements can easily be performed over a distance of about 1 kilometer.

6. Discussion

The results demonstrate, that it is possible to detect inhibitors of the enzymes acetyl- and butyrylcholine esterase under the applied conditions on-line, i.e. continuously over a period of several days. The use of optical fibers allows measurements over large distances. With the novel and robust fiber-optic 3-Lambda-Photometer, which is equipped with light emitting diodes, photodiodes and optical plastic fibers, it is possible to measure over a distance of approximately 50 meters from the place where the samples are drawn. Distances of up to one kilometer can be acchieved if lasers and glass fibers are used.

The detection limits for paraoxon of 200 nM (= 55 µg/l) and for BW 284 C51 of 10 nM (= 5.7 µg/l) are acceptable, whereas the detection limit of 1 µM (= 0.26 mg/l) for pyridostigmine is not. However, the sensitivity of the method can be increased. It was shown that the optimal temperature for the inhibition of acetylcholine esterase with

paraoxon is about 30 °C [17]. All measurements presented here were performed at room temperature. Not only the temperature, but also the flow-rate could be increased to enhance the sensitivity of the method towards the inhibitors (Figs. 11 & 15). However, this would lead to an additional consumption of enzyme substrate and a shorter lifetime of the immobilized enzyme. The mixing ratio of substrate:buffer:inhibitor solutions could also be changed in favour of the inhibitor solution. It is also known, that choline esterase enzymes from different sources show differing sensitivities towards the various inhibitors [20]. For the detection of a certain pesticide it would be possible to choose another more appropriate enzyme. Finally the signal/noise ratio of the measuring system can be improved through optical and electronical refinements. With such an optimized system, very small signal changes can be recognized and the detection limits could be lowered by one or two orders of magnitude.

For the determination of BW 284 C51 and pyridostigmine, no data were available from the literature. The determination of paraoxon has been described several times. In a radiometric method with an incubation time of 60 minutes and a total time requirement of at least two hours the detection limit was as low as 4.29×10^{-10} M paraoxon [11]. In a colorimetric method the incubation time was two hours and the whole procedure lastet ca. 2.5 hours [11]. A very tedious procedure with an immunoassay coupled to an enzymatic reaction (total time consumption ca. 2.5 hours) resulted in a detection limit of 1 nM paraoxon [11].

The method presented here doese not require radioactivity or antigenes. A detection limit of 200 µM paraoxon after a measuring time of 21 minutes seems to be very acceptable especially in view of the possible enhancement through opto-electronics. It would be no problem to measure the signal changes caused by the inhibition process after one hour and to increase the sensitivity. For practical purposes, a method which gives a signal change after one hour is still more useful than a method which requires two hours, especially in a pesticide alarm system.

In a flow injection analysis system (FIA) a rapid and somewhat sensitive determination of paraoxon was possible [17]. Using this method, 60 samples per hour with a detection limit of 400 nM paraoxon or 30 samples per hour (in the stopped-flow mode) with a detection limit of 8 nM were measured. Both, the inhibitor and the substrate solutions, were injected into a buffer carrier stream. The synthetic substrate was hydrolyzed in an enzyme reactor and the product reacted with a second reagent to form a dye whose absorption was measured at 500 nm. According to the authors, the rapid determination of the pesticide was possibl because inhibitor and substrate reached the enzyme at the same time and a competition in binding to the active site occured. No long incubation times were necessary. This method seems to be applicable to our system and will be investigated in the near future. A rapid analysis of small sample volumes is advantageous in many cases, although a more complex and expensive instrumentation is required.

The method proposed in this work could be used for continuously monitoring a drinking or ground water reservoir. The signal change produced for example by an organophosporus or carbamate pesticide may serve as an alarm to switch of the water supply or to start more detailed investigations, such as qualitative and quantitative analysis or search for the origins of the pesticides. It would also be of interest to test the response of the system towards the so-called nerve gases, since these substances are closely related to the organophosphorus pesticides. It is known that these chemical warfare agents are very potent inhibitors of the enzymes acetyl- and butyrylcholine esterase. Therefore these compounds must be detectable with our system. The identification of nerve gases over large distances could provide a simple and sensitive alarm system, not only in cases of war but also in case of an accident in the production, transport or destruction of such substances.

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Figures

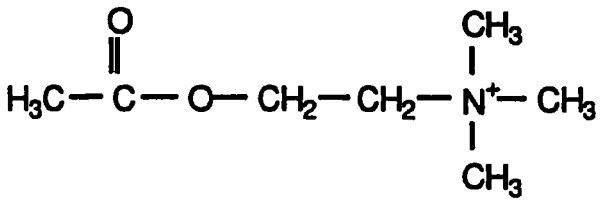

Fig. 1. Acetylcholine

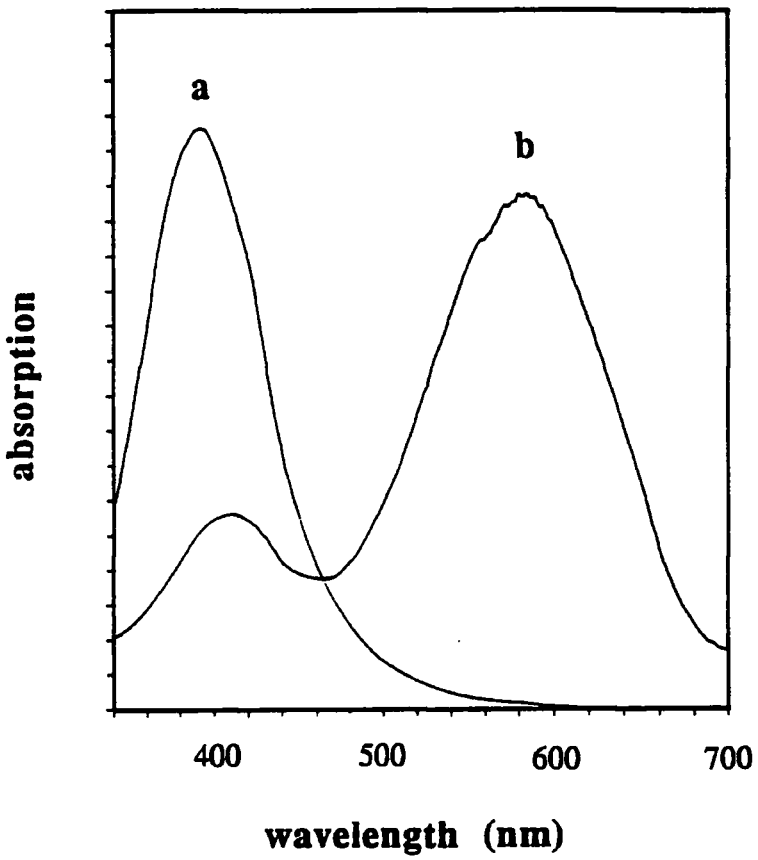

Fig. 2. Absorption spectra of Substrate A (a) and its product of hydrolysis (b) at pH 7.5

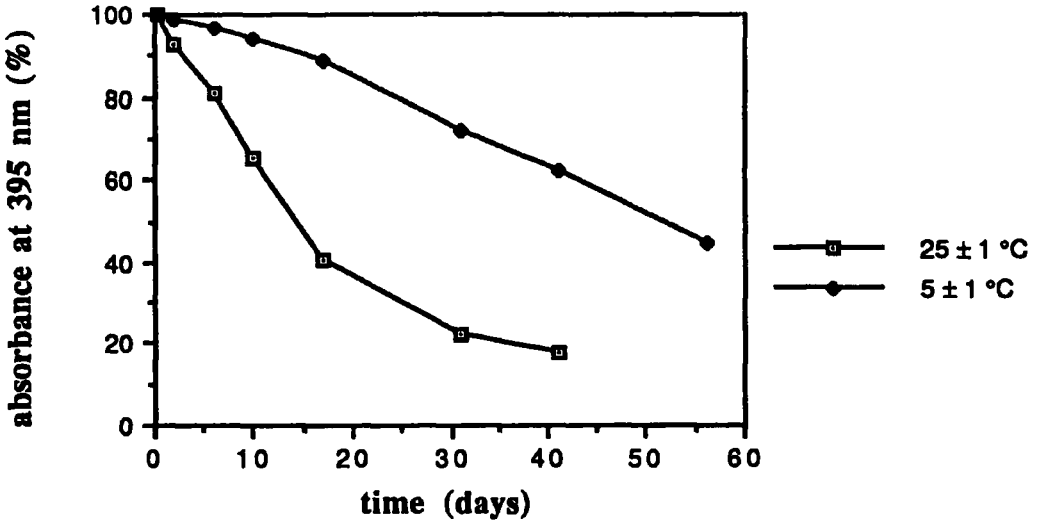

Fig. 3. Stability of Substrate A in destilled water at 25 ± 1 °C and at 5 ± 1 °C in the dark

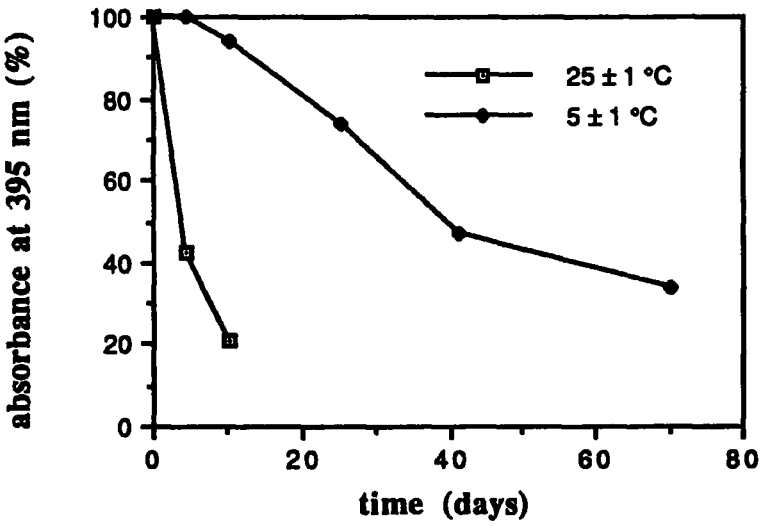

Fig. 4. Stability of Substrate B in destilled water at 25 ± 1 °C and at 5 ± 1 °C in the dark

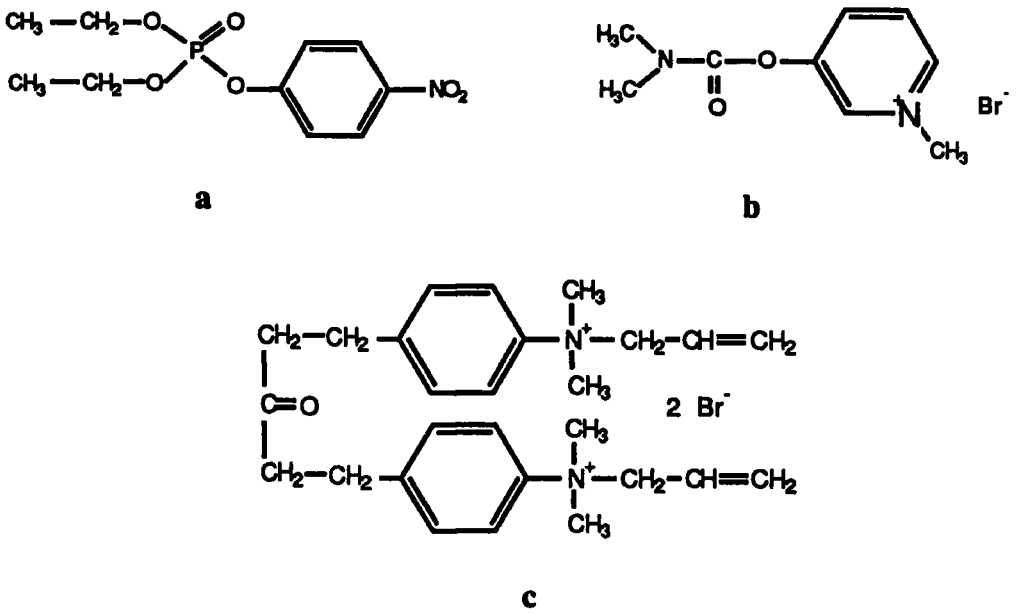

Fig. 5. (a) Paraoxon; (b) pyridostigmine bromide; (c) BW 284 C51

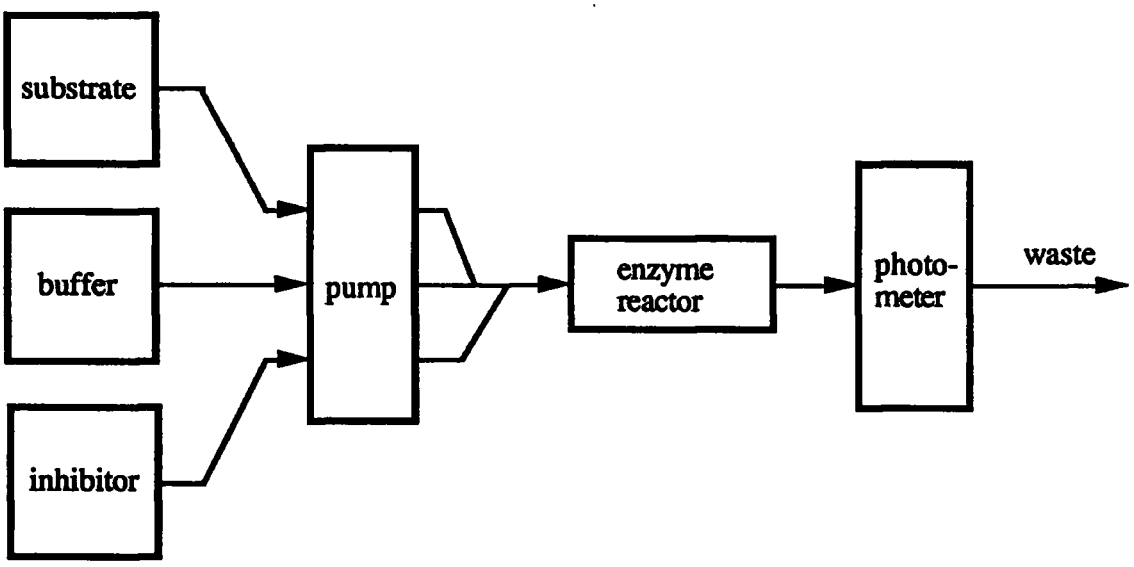

Fig. 6. Experimental set-up for measurements with an UV/VIS-spectrophotometer

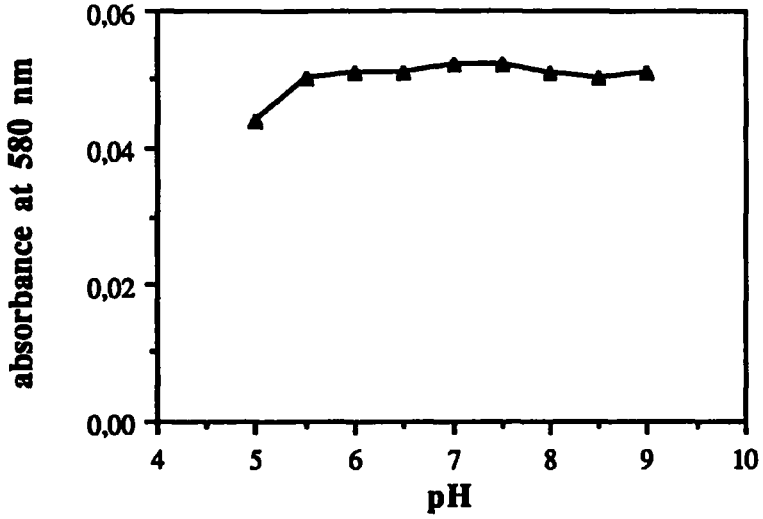

Fig. 7. pH-dependence of the enzymatic hydrolysis of Substrate A at a flow-rate of 0.58 ml/min.

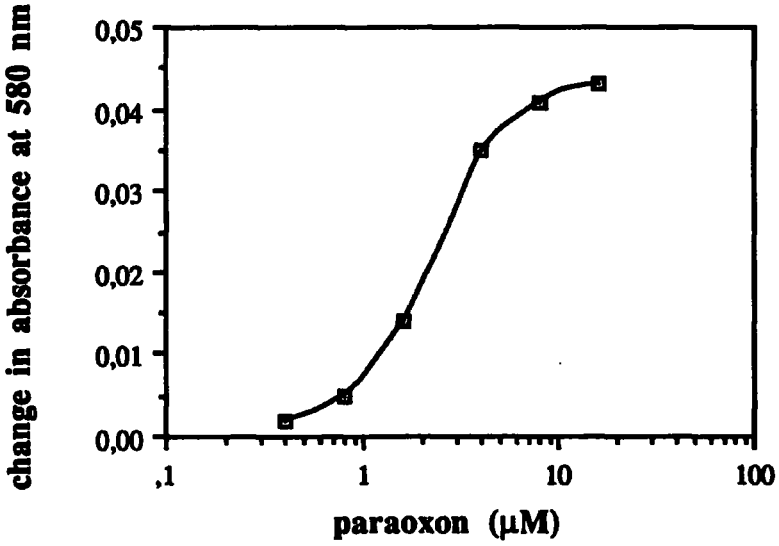

Fig. 8. Inhibition of acetylcholine esterase with paraoxon - calibration graph (signal changes after 21 min; pH 7.5; flow-rate: 1.8 ml/min)

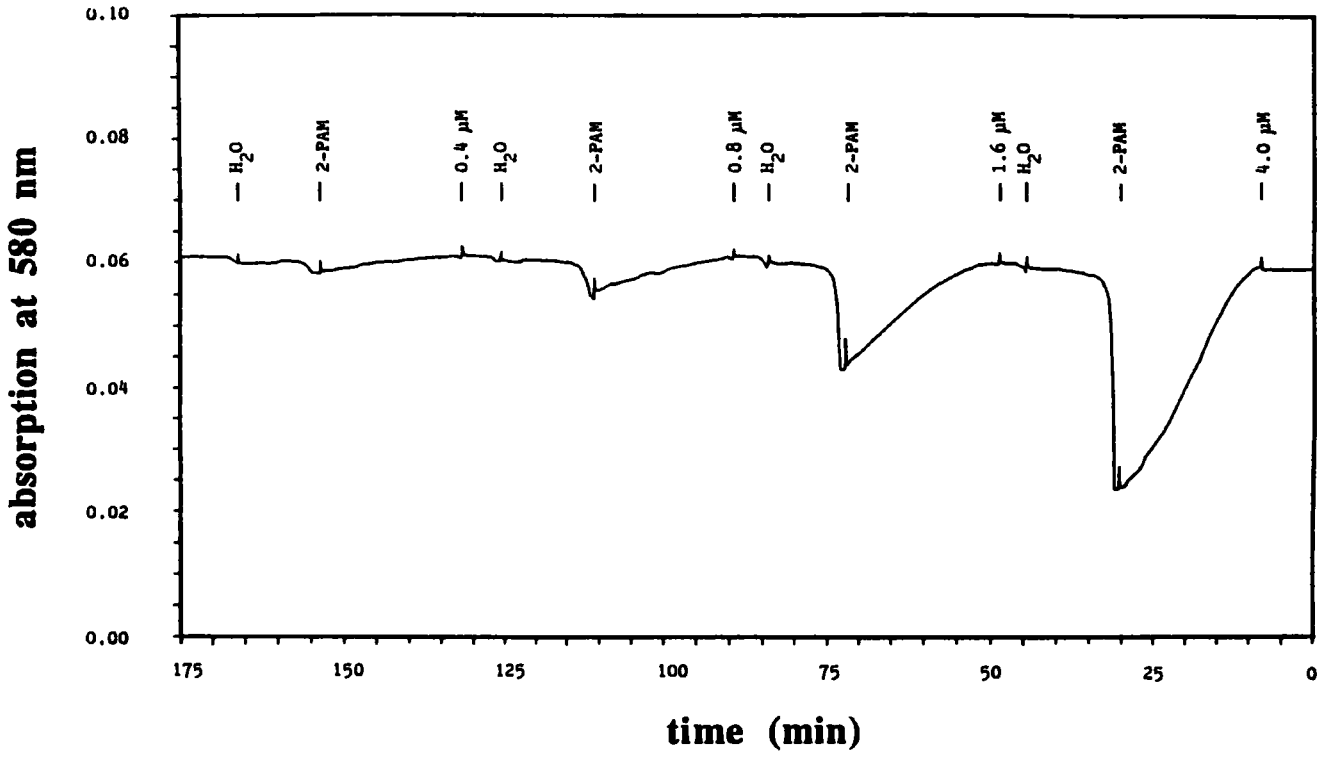

Fig. 9. Inhibition of acetylcholine esterase with paraoxon - time dependence (pH 7.5; flow-rate: 1.8 ml/min)

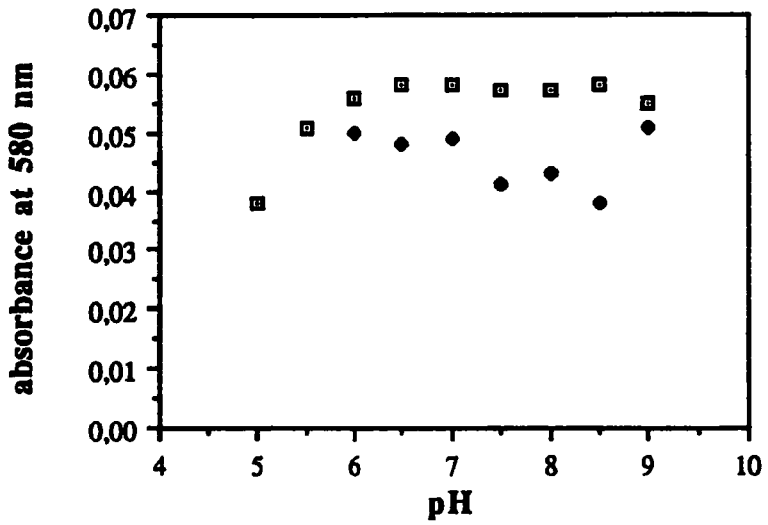

Fig. 10. Inhibition of acetylcholine esterase with paraoxon - pH-dependence (signal changes after 21 min; flow-rate: 1.46 ml/min)

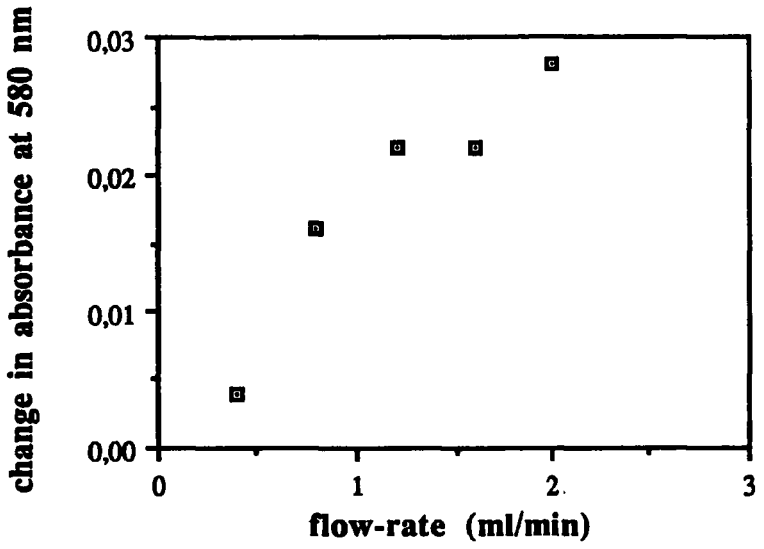

Fig. 11. Inhibition of acetylcholine esterase with paraoxon - flow-rate dependence (signal changes after 21 min; pH 7.5)

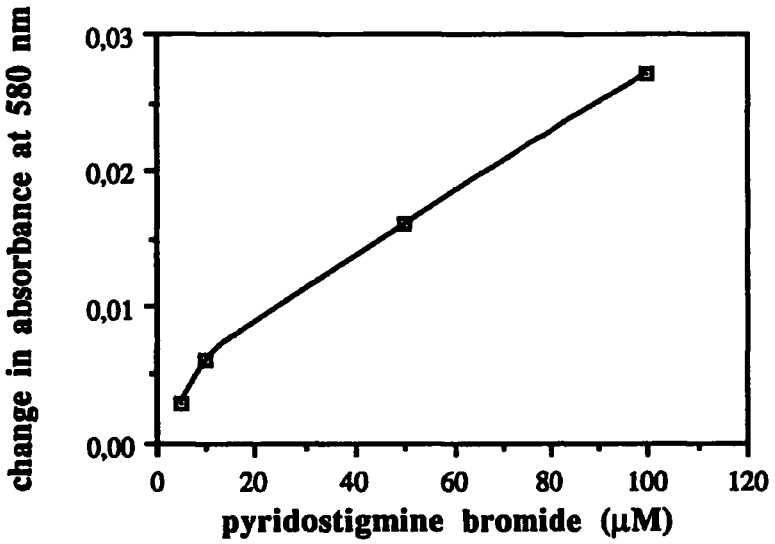

Fig. 12. Inhibition of acetylcholine esterase with pyridostigmine - calibration graph (signal changes after 21 min; pH 7.5; flow-rate: 1.8 ml/min)

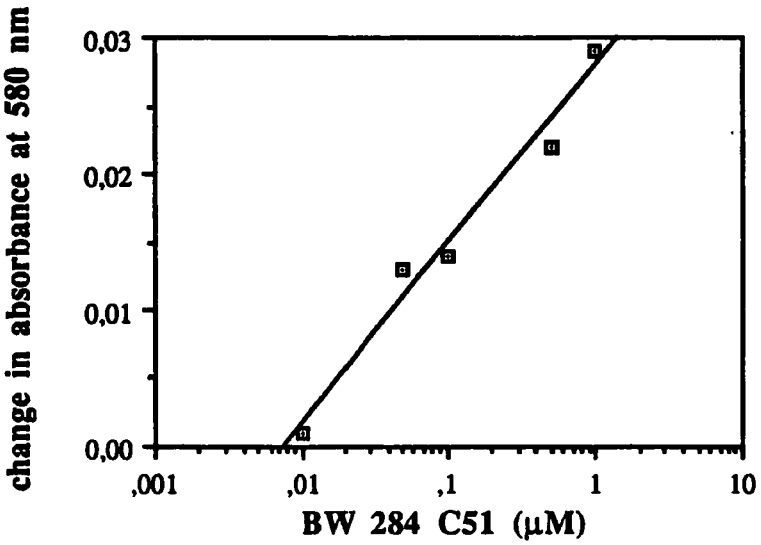

Fig. 13. Inhibition of acetylcholine esterase with BW 284 C51 - calibration graph (steady-state response; pH 8.0; flow-rate: 1.2 ml/min)

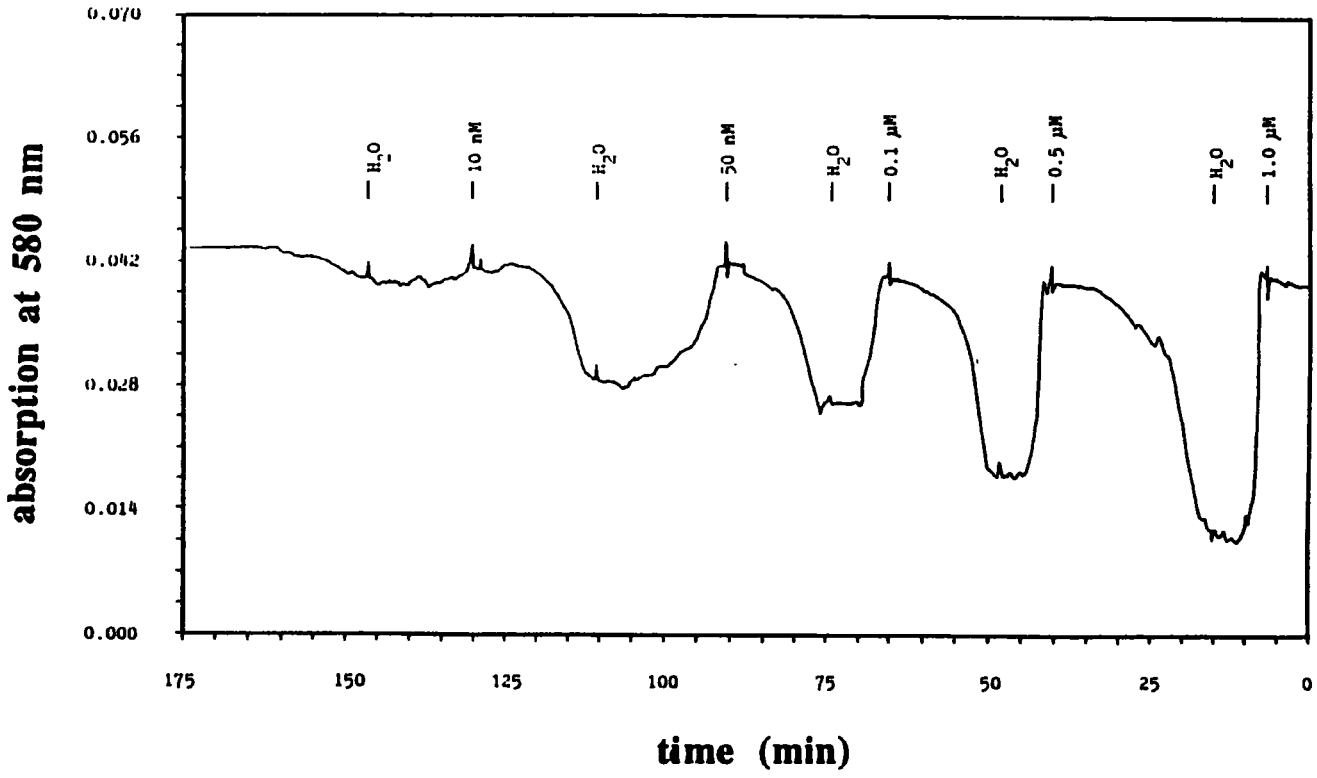

Fig. 14. Inhibition of acetylcholine esterase with BW 284 C51 - time dependence (pH 8.0; flow-rate: 1.2 ml/min)

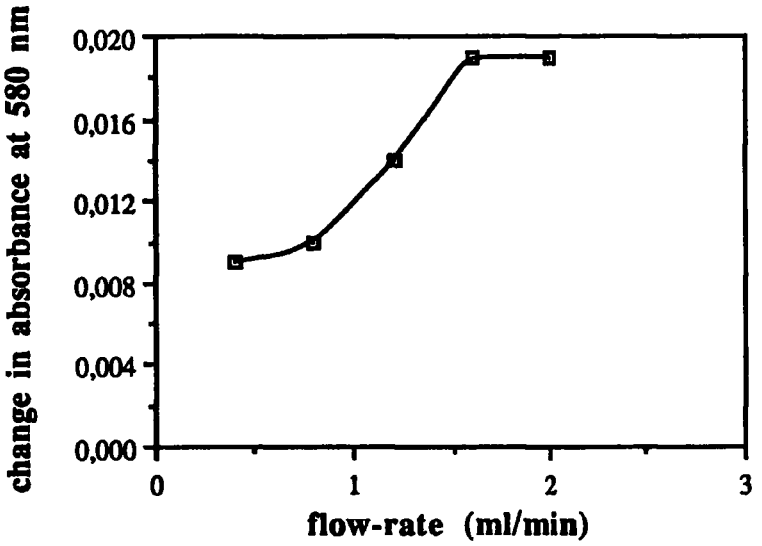

Fig. 15. Inhibition of acetylcholine esterase with 0.1 µM BW 284 C51 - flow-rate dependence (steady-state response; pH 8.0; flow-rate: 1.2 ml/min)

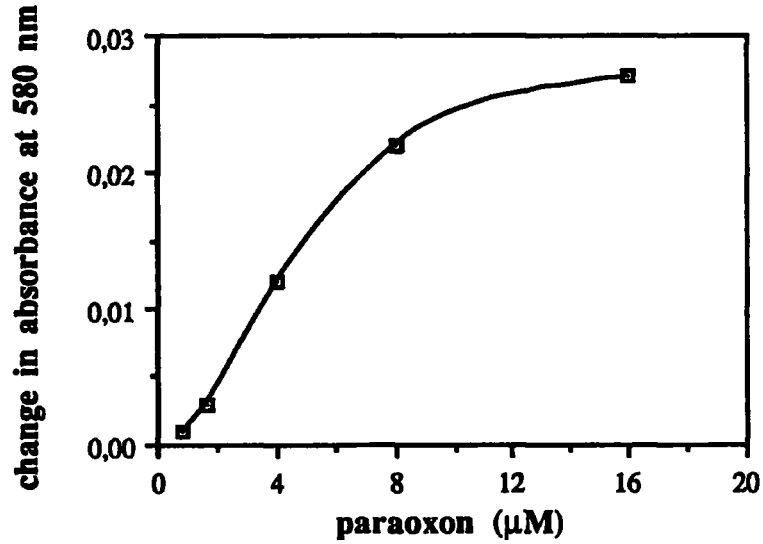

Fig. 16. Inhibition of acetylcholine esterase with paraoxon - calibration graph (signal changes after 21 min; pH 7.5; flow-rate: 1.8 ml/min)

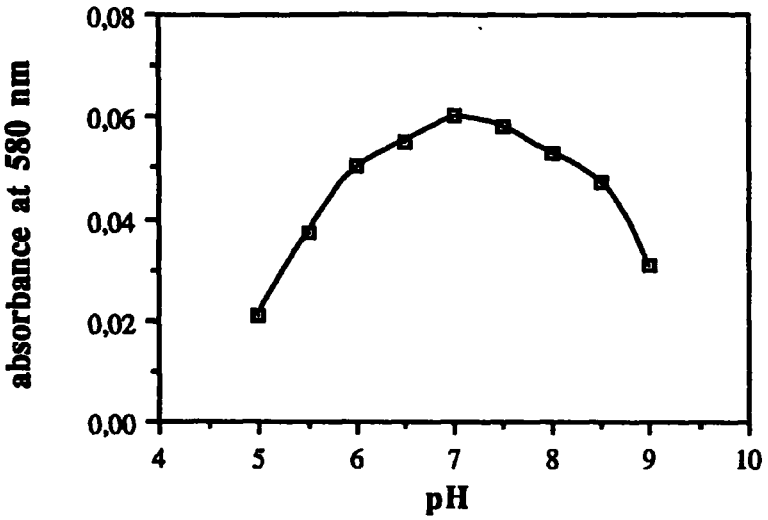

Fig. 17. pH-dependence of the hydrolysis of Substrate B by butyrylcholine esterase immobilized on aminopropyl-CPG with glutaraldehyde (flow-rate: 1.8 ml/min)

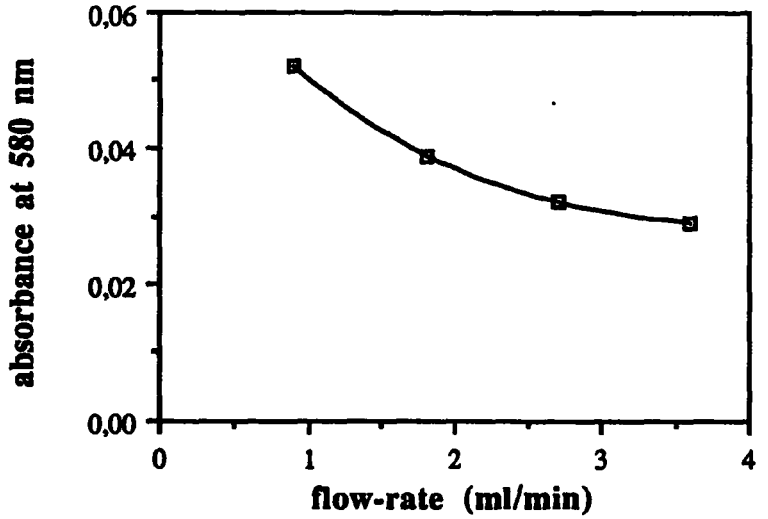

Fig. 18. Flow-rate dependence of the hydrolysis of Substrate B by butyrylcholine esterase immobilized on aminopropyl-CPG with glutaraldehyde (pH 7.5)

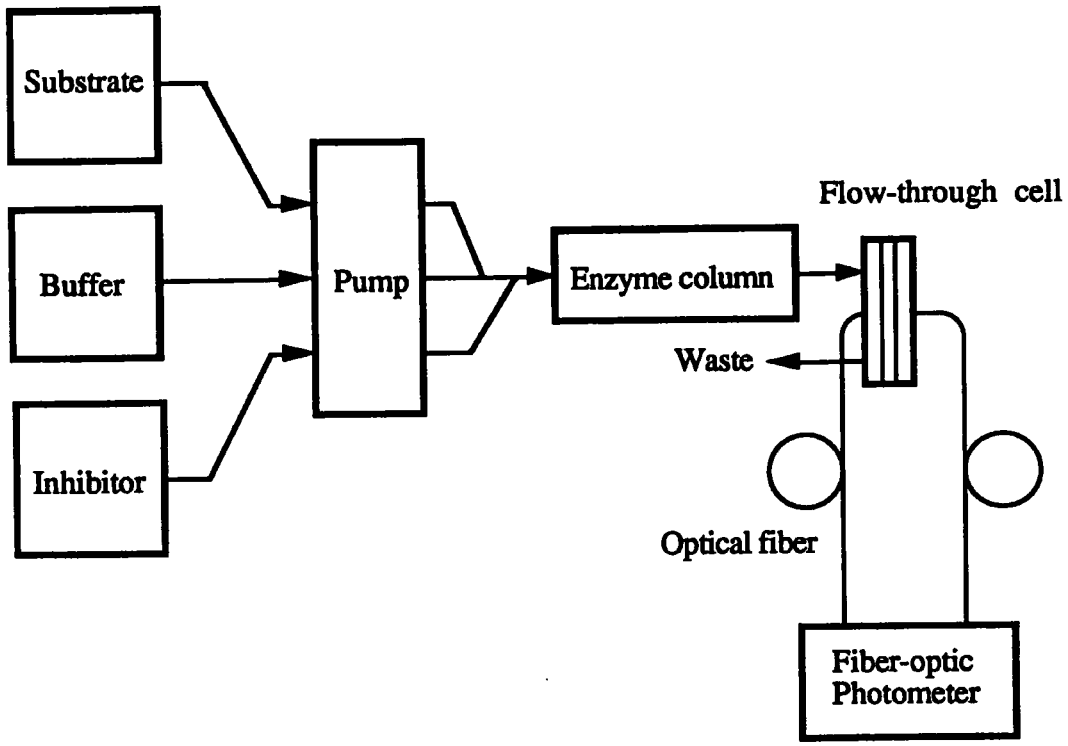

Fig. 19. Experimental set-up for measurements with a fiber-optic photometer

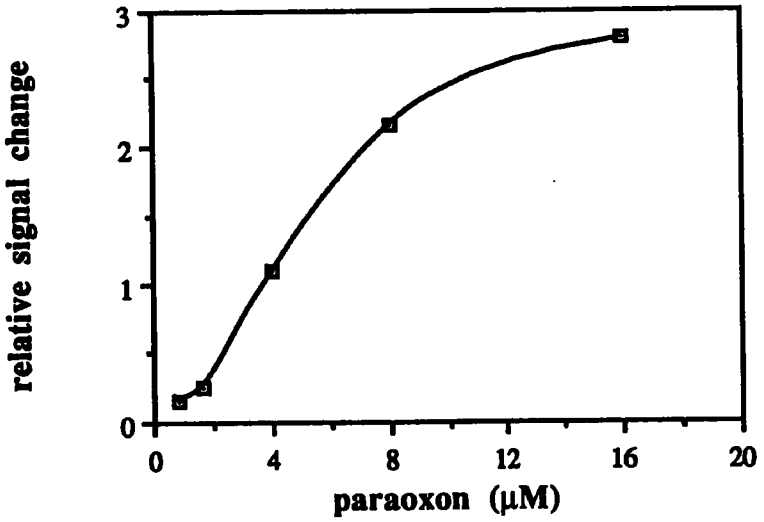

Fig. 20. Inhibition of acetylcholine esterase immobilized on VA-Epoxy-Biosynth with paraoxon (signal changes after 21 min; pH 7.5; flow-rate: 1.8 ml/min)

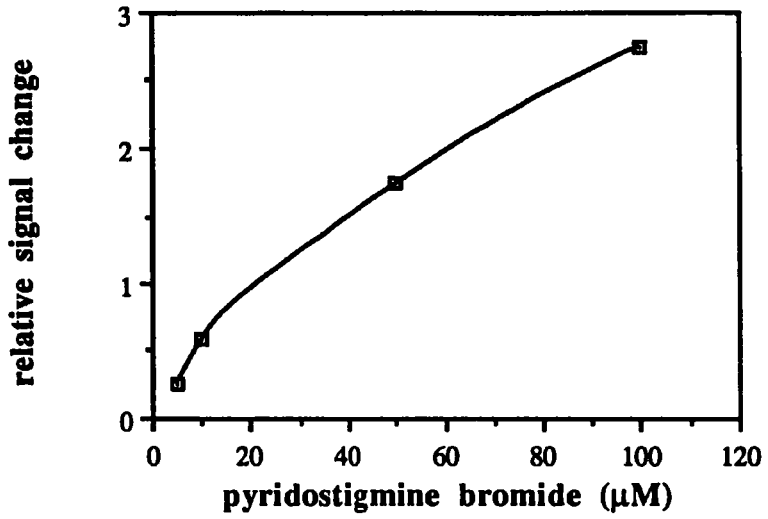

Fig. 21. Inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with pyridostigmine (signal changes after 21 min; pH 7.5; flow-rate: 1.8 ml/min)

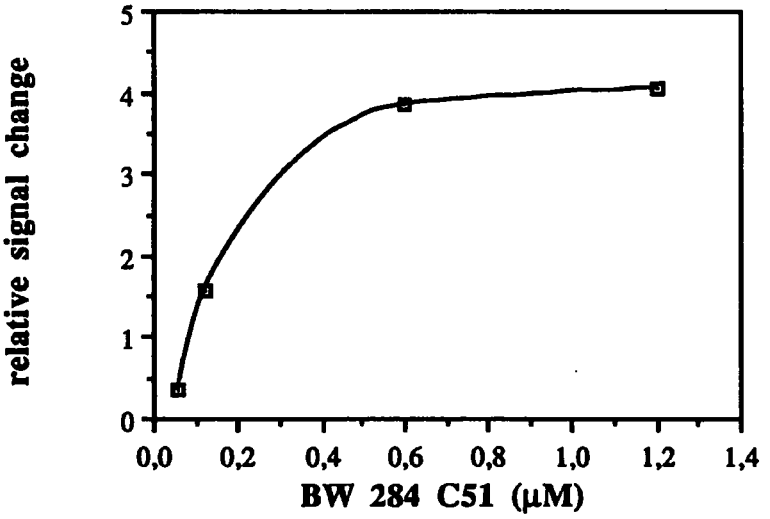

Fig. 22. Inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with BW 284 C51 (steady-state response; pH 7.5; flow-rate: 1.8 ml/min)

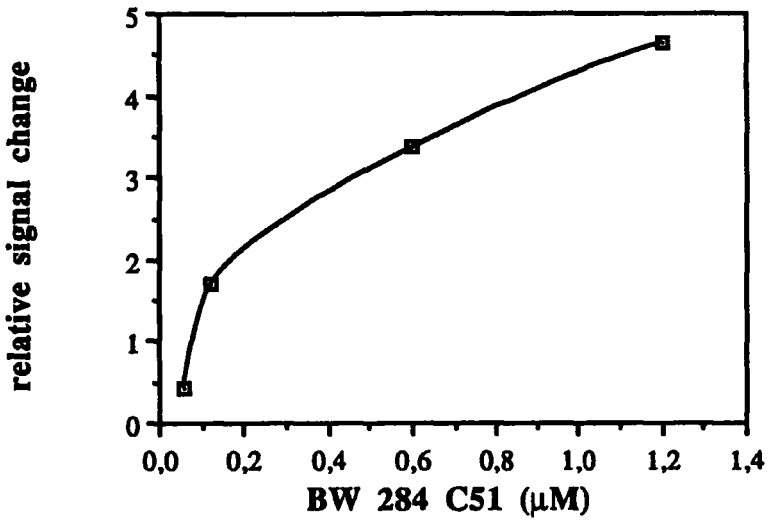

Fig. 23. Inhibition of acetylcholine esterase immobilized on aminopropyl-CPG with BW 284 C51 (steady-state response; pH 7.5; flow-rate: 1.8 ml/min)