0273-2289/00/89/0217/$13.50

Amperometric Biosensors Based on Microflow Injection System

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Abstract

Novel electrochemical cells based on a microflow system combined with amperometric enzyme electrodes were developed and served for quantitative determination of various compounds, such as organophosphates and lactose. The resulting biosensors are selective and efficient owing to immobilization of the sensing elements on the electrodes. The sensors are easy to operate, and the procedures are rapid, accurate, reproducible, and inexpensive, requiring neither special skills and training nor complicated instrumentation. The use of a microflow cell ensures the continuous flux of a new substrate, thus preventing the accumulation or adsorption of products to the electrode. Miniaturization of the sensor has two main advantages: (1) it is easy to carry and therefore can be used outdoors as well, and (2) it allows working with low volumes of compounds and reagents, which is highly important when dealing with hazardous compounds.

Index Entries: Microflow; amperometric biosensor; injection; electrode.

Introduction

The ability to identify selected compounds qualitatively as well as quantitatively is one of the primary concerns of environmentalists. Along with many advantages, progress in technology has evoked crucial environmental and ecological problems. Vast numbers of toxic compounds are released into the atmosphere during industrial and agricultural manufacturing processes, threatening human health and welfare. However, the immense progress in scientific research and medicine have enabled us to relate certain illnesses and defects to one or more biochemical components. Therefore, the quantitative identification of certain compounds is of great importance.

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Biosensors are an attractive as well as powerful tool to achieve this goal. They allow "on-the-spot" detection of desired factors such as chemical residues, nutritive compounds, bacterial contaminators, and antibiotics *(1)*. Bioelectrochemical sensors generally consist of a biologically sensitive component, such as an enzyme, an antibody, and a receptor attached to an electrode, and a suitable substrate. During the biochemical reaction, ions or electroactive substances may be produced *(2)*. By using well-established electrochemical techniques, the biochemical response is converted into an amplified, quantifiable electrical signal.

The natural choice of bioactive component in a bioelectrochemical sensor would be the enzyme because it comprises high selectivity and sensitivity. Redox enzymes are especially useful. The electrons transferred by these enzymes can be trapped and shuttled through an electric circuit, resulting in a current proportional to the enzymatic reaction *(3,4)*.

Major efforts are being made to develop a small biosensor that would allow decentralization of analytical measurements from the laboratory to the field. This kind of biosensor should be inexpensive and simple to operate by a nonqualified person. It should provide immediate results and be amenable to mass production. Miniaturization of biosensors may provide another advantage: it offers the ability to work with small amounts of materials, which is of great importance while working with hazardous compounds.

We have developed a microthin layer flow system in which the enzyme is immobilized onto the electrode and a tested solution passes along the electrode's surface. The flow rate could be optimized according to the system's characteristics in order to obtain maximal reaction efficiency together with minimal product accumulation. In this way we reduce the amount of enzyme needed for detection and the time response, because the diffusion distance is decreased. We have tested several configurations of the electrochemical cells. In two cases, a homemade flow cell and the electrodes used were surface-modified screen-printed electrodes (SPEs) *(5)*. In a third system, a commercial electrochemical flow cell with a small modified glassy carbon electrode was used. In all cases, the working electrode was covered with an enzymatic membrane. The resulting biosensors are cheap, small, efficient, reliable, disposable, and stable for a relatively long time.

We describe three distinct examples of the usage of enzyme-based biosensors:

1. A direct biosensor for determining the presence of parathion by measuring enzymatic activity.
2. A biosensor based on inhibition of enzymatic activity for the detection of organophosphates.
3. A biosensor based on a three-enzyme cascade reaction for the detection of lactose in milk and milk products.

The first and second systems deal with a group of substances known as organophosphate compounds. A variety of these compounds have been and still are widely used as insecticides in modern agriculture *(6)*. The acute toxicity of organophosphate compounds to the human nervous system results from its strong binding to the enzyme acetylcholine esterase (AChE), forming a very stable complex that inhibits enzymatic activity. Detection of organopollutants that threaten living organisms is a crucial civil issue, not to mention a military concern because they are used in chemical warfare *(7)*.

The third biosensor concerns the identification and determination of a naturally occurring ingredient: lactose. The major source of lactose in food industries is cow's milk. It is a disaccharide that contains two different monosaccharide units: D-glucose and D-galactose. The normal concentration of lactose in milk is about 4 to 5% (40–50 mg/mL), and only small amounts of nonlactose carbohydrates have been found. Lactose is widely used in baking ingredients, ready-to-eat products, and commercial milk formulas for infants. Because it is a basic component in various kinds of edible products, its amount determines the nutritive value of products. Also note that for 5–15% of the population, the consumption of lactose is forbidden because these individuals lack one of the enzymes (mostly lactase) along the digestion cascade of this sugar. To these people consumption of lactose might be harmful. Therefore, the development of an inexpensive and simple sensor to determine lactose concentration in various edible components is worthwhile.

Several procedures are currently available for lactose determination. Among the physical methods are gas, liquid, and high-pressure liquid chromatography *(8)*; polarimetry *(9)*, and gravimetric analysis *(10)*. The major disadvantages of these methods are that they are time-consuming, relatively insensitive, require rather complex sample preparation, and expensive.

Materials and Methods

Chemicals and Reagents

Pseudomonas sp. was isolated from the Upper Galilee soil *(11)*, contaminated with residues of organophosphate pesticides, and obtained from Migal-Galilee Technology Center (Kiryat Shmona, Israel). Parathion hydrolase from *Pseudomonas* sp. was purified by successive precipitation of cell extract after sonication with protamine sulfate and ammonium sulfate, followed by dialysis against 50 m*M* Tris buffer (pH 8.8) and fractionation by DE-cellulose (DE52, Whatman, England) *(12)*. Acetylthiocholine chloride (ATCh), pyridine 2-aldoxime methiodide (PAM), and acetylcholine esterase (AChE) type V-S from electric eel were purchased from Sigma (St. Louis, MO). Dimethyl2,2-dichlorovinyl phosphate (DDVP) was obtained from Machteshim (Israel), and $K_3[FeCN_6]^{-3}$ and $K_4[Fe(CN)_6]^{-2}$ were from Merck. Nylon membranes (6.6) (Immunodyne® ABC, 5- and 3-μm cutoff) were purchased from Pall. β-Galactosidase (EC 3.2.1.23, from *Escherichia*

coli, type VIII; 520 U/mg of solid), glucose oxidase (GOD) (EC 1.1.3.4, from *Aspergillus niger*, type VII; 151 U/mg of solid), horseradish peroxidase (HRP) EC 1.1.7, type VI; 210 U/mg of solid), water-polyethyleneimine (PEI) solution (50%), and β-lactose were obtained from Sigma. Water-GA solution (50%) was purchased from Fluka. Raw milk samples were obtained from the Volcanic Agriculture Institute. All other reagents and buffers were analytical reagent grade. Any reagent or electrolyte solution was prepared using double-deionized water.

Determination of Organophosphates

The electrochemical system was based on amperometric measurements. The working apparatus was composed of SPEs including a working electrode, a reference electrode, and a counterelectrode placed in a homemade microflow cell (cell volume of 30 μL). The cell was washed with buffer solution using a 1-mL syringe. The enzymes were attached to the working electrode via an activated membrane. The substrate solution was injected into the cell through a 5-μL injection loop. The use of a flow cell system allowed fast repetitive measurements of an enzymatic reaction as the product was removed and the substrate readded. The flow rate was optimized for each system. The electrodes were connected to a computer-controlled potentiostat. The potentiostat and the software used were from BAS. Bioanalytical Systems. Figure 1 illustrates the electrochemical cell.

Fabrication and Surface Modifications of SPEs

SPEs were obtained from EmTech (Israel). The electrodes were composed of a carbon ink working electrode, a Ag/AgCl reference electrode, and a carbon ink counterelectrode. The electrodes were printed on a mylar layer. Two different biosensors using the same apparatus with slight modifications are described next.

Parathion Sensor

Two hundred microliters of 0.5% PEI in water was spread on the SPEs. After 20 min at room temperature, the electrodes were washed in distilled water and placed in a 2.5% GA in phosphate buffer, pH 7.0, for 1 h. The electrodes were then washed in phosphate buffer and 5 μL of parathion hydrolase solution (600 U/mg) was spread on the electrode surface. The electrodes were left for 2 h at room temperature and then washed in phosphate buffer. The modified SPEs were deposited in the lower part of the flow cell. Tris-HCl (0.1 *M*) flowed continuously and various concentrations of parathion were injected.

DDVP Sensor

The DDVP biosensor was designed to measure the initial activity of the enzyme AChE and the subsequent decrease in enzymatic activity after exposure to DDVP *(13)*. AChE was dissolved in 0.1 *M* phosphate buffer, pH 7.5, to a final concentration of 0.1 mg/mL. The enzyme was attached to

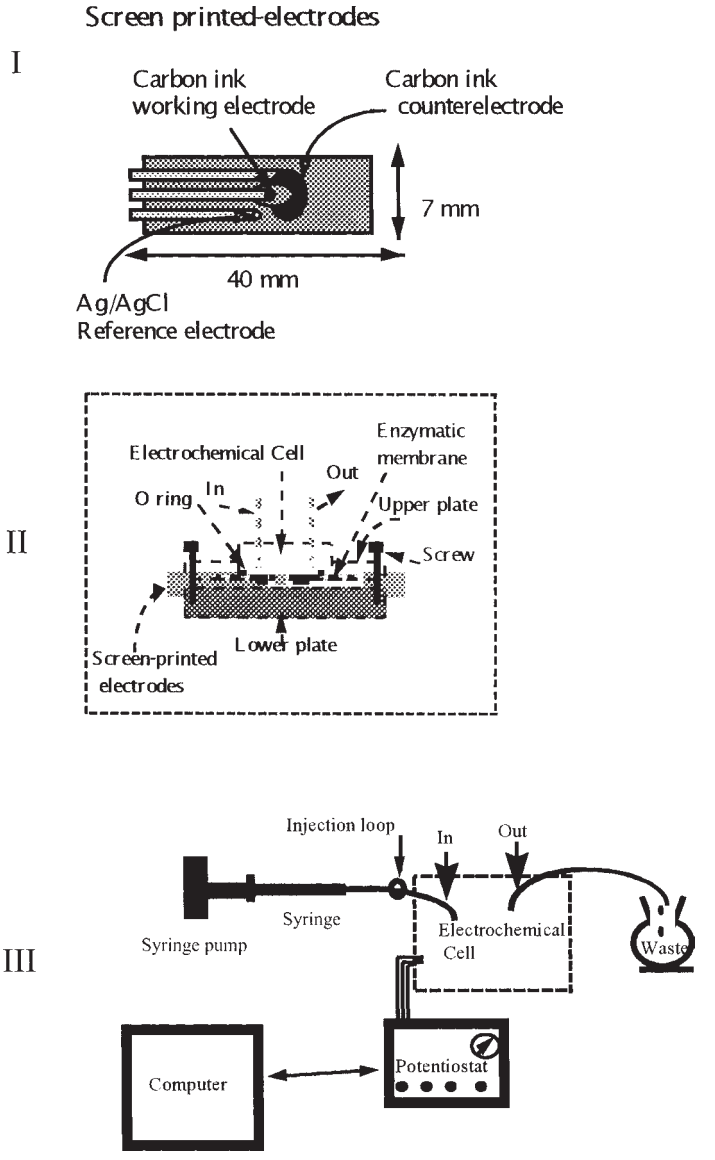
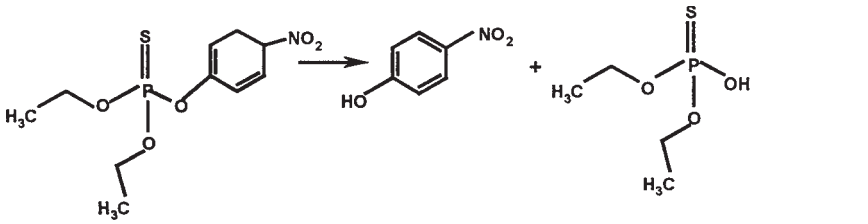


Fig. 1. Electrochemical cell setup: I, screen-printed electrodes; II, electrochemical microflow cell; III, entire measuring system.

the working electrode via an activated nylon membrane (Immunodyne ABC, 5- and 3-μm cutoff, from Pall). The membrane was cut into discs (6 mm in diameter). Five microliters of the AChE solution (100 μg/mL in 0.1 *M* phosphate buffer, pH 8.0) was applied onto each disc of the Immunodyne membrane. After air-drying, the discs were transferred to a blocking solution of 0.1 *M* phosphate buffer, pH 7.5, containing 0.1 *M* glycine and then washed with 0.1 *M* phosphate buffer. The discs were stored at 4°C in the same buffer solution and were active for several months.



O, *O*-diethyl *O*-4-nitrophenyl phosphorothioate (parathion) | *p*-nitrophenol (PNP) | di-ethylthiophosphoric acid (DEPA)

Scheme 1. Decomposition of parathion by parathion hydrolase.

To measure the organophosphates, the discs was placed on the SPEs in the flow cell. Measurements were done at 300 mV vs Ag/AgCl. Phosphate buffer (0.1 *M*) solution containing 0.1 m*M* $K_3Fe(CN)_6$ flowed continuously and 5 µL of the substrate ATCh, or the inhibitor DDVP, or the activator PAM were injected.

Lactose Sensor

The flow cell used for lactose determination was a Unicell manufactured by BAS. It contained a 2-mm-diameter glassy carbon electrode (MF-1025) and auxiliary and reference electrodes block (MF-1005) with four 2-mL round gaskets (MF-1059). The total electrochemical cell volume was 8 µL.

Preparation of Electrode and Working Procedure

Three enzymes were immobilized on the glassy carbon electrode via cross-linkage with PEI *(14)*. The immobilization solution consisted of 2 mg/mL of β-galactosidase, 1 mg/mL of GOD, 1 mg/mL of HRP, 0.25% PEI, 0.025% glutaraldehyde, and 0.02 *M* phosphate buffer, pH 7.5. Five microliters of this solution was successively applied on the electrode, with drying intervals between additions. The electrodes were left to dry completely following the last addition and then washed and stored in 0.1 *M* phosphate buffer, pH 7.5, at 4°C.

Measurements were taken in a solution composed of 0.05 *M* phosphate buffer (pH 7.0), 0.05 *M* KCl, 2 m*M* MgCl, and 5 m*M* $K_4Fe(CN)_6$ at a flow rate of 50 mL/min. The working potential was –200 mV vs the reference electrode in the flow cell.

Results and Discussion

Quantitative Determination of Parathion

A direct sensor was designed to identify and quantify parathion in solution. Parathion is hydrolyzed by the enzyme parathion hydrolase according to the reaction in Scheme 1. Parathion was injected into the flow

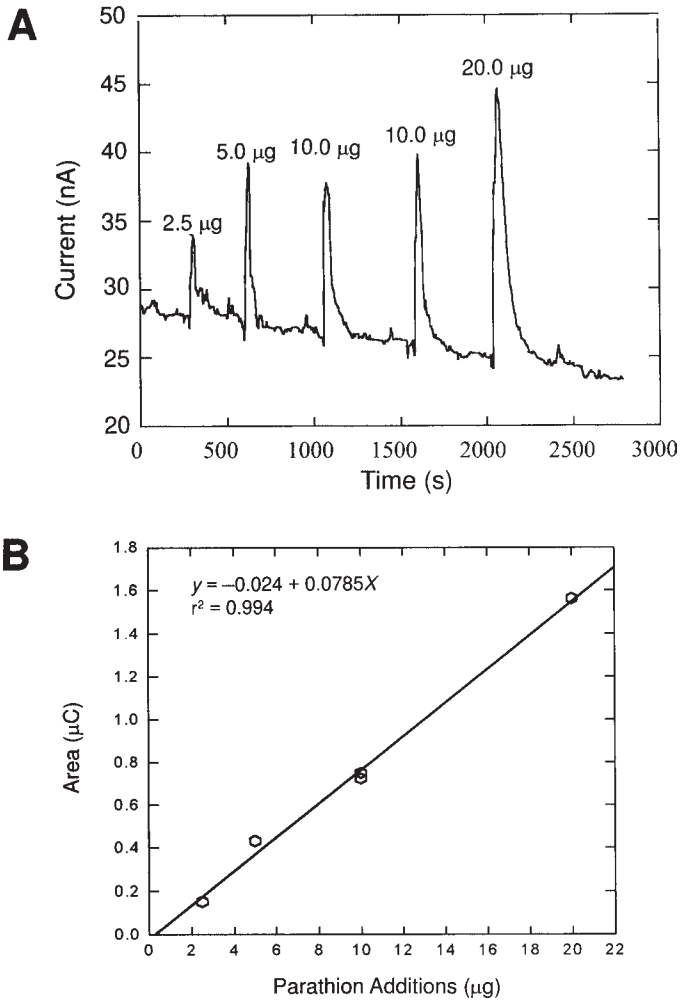


Fig. 2. **(A)** Time-dependent response of modified electrode to parathion. Flow rate, 0.5 mL/min. **(B)** Calibration curve for parathion.

system and the change in the current owing to the enzymatic hydrolysis in the electrochemical cell was monitored. The *p*-nitrophenol formed was detected electrochemically at anodic potentials. We have characterized and optimized the *p*-nitrophenol current signal and have chosen 900 mV vs Ag/AgCl as the working potential.

In a set of experiments, various amounts of parathion were added to the electrochemical cell. Optimal flow rate was found to be 0.5 mL/min and allowed enough time for the enzyme to react with the substrate and for the product to diffuse toward the electrode. Figure 2A depicts the correlation between the amount of parathion and the current developed in response. Each peak represents the increase in current followed by the addition of parathion and the subsequent decrease after cell wash with the buffer solution. The area under each peak was calculated by integration of the

signal and reflected product concentration. Successive additions of different amounts of parathion resulted in a linear correlation between the amount of parathion and the current response. Figure 2B gives a calibration curve for parathion.

It is evident that this biosensor can trace small amounts of a desired substrate by using a naturally occurring hydrolysis reaction that produces an electrochemically active measurable product. When dealing with hazardous environmental pollutants, the ability to detect low concentrations is extremely important. Moreover, the flow system ensures high accuracy and allows repetitive measurements. Because the biosensor is small and its price is low, it is a useful as well as convenient candidate for an outdoor on-the-spot detector.

Quantitative Determination of DDVP

Biosensors for the detection of organophosphate compounds *(15,16)* including techniques based on the inhibition of the enzyme AChE are described in the literature *(17–19)*. The innovation of the present study is the use of SPEs in a microflow system. The sensor is based on the following reactions:

$$(CH_3)_3N^+CH_2CH_2S - OCCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH$$

$$2(CH_3)_3N^+CH_2CH_2SH + 2[Fe(CN)_6]^{-3} \rightarrow (CH_3)_3N^+CH_2CH_2S - SCH_2CH_2N^+(CH_3)_3 + 2[Fe(CN)_6]^{-4}$$

$$[Fe(CN)_6]^{-4} \rightarrow [Fe(CN)_6]^{-3} + e^-$$

The thiocholine formed by the enzymatic reaction reduces the $[FeCN_6]^{-3}$ to $[FeCN_6]^{-4}$, which is oxidized back to $[FeCN_6]^{-3}$ at the electrode, generating the amperometric signal. Various concentrations of the substrate acetylthiocholine were added and the electrode response was monitored (Fig. 3A). Integration of the signals resulted in a calibration curve (Fig. 3B).

The reaction between the inhibitor and the enzyme is as follows:

$$E - OH + (RO) - \underset{\displaystyle (RO)}{\overset{\displaystyle O}{\overset{\displaystyle \|}{\underset{\displaystyle |}{P}}}} - X \xrightarrow{K_I} (RO) - \underset{\displaystyle (RO)}{\overset{\displaystyle O}{\overset{\displaystyle \|}{\underset{\displaystyle |}{P}}}} - OE + HX$$

A complex is formed between the serine group on the enzyme and the phosphate group of the inhibitor. The rate of complex formation is given in the following expression:

$$\frac{d[EI]}{dt} = K_I\{[E] - [EI]\}\,[I]$$

in which $[I]$ is the inhibitor concentration; $[E]$ is the enzyme concentration prior to exposure to the inhibitor; and $[EI]$ is the complex concentration. Integration of this equation will result in a linear dependence of complex

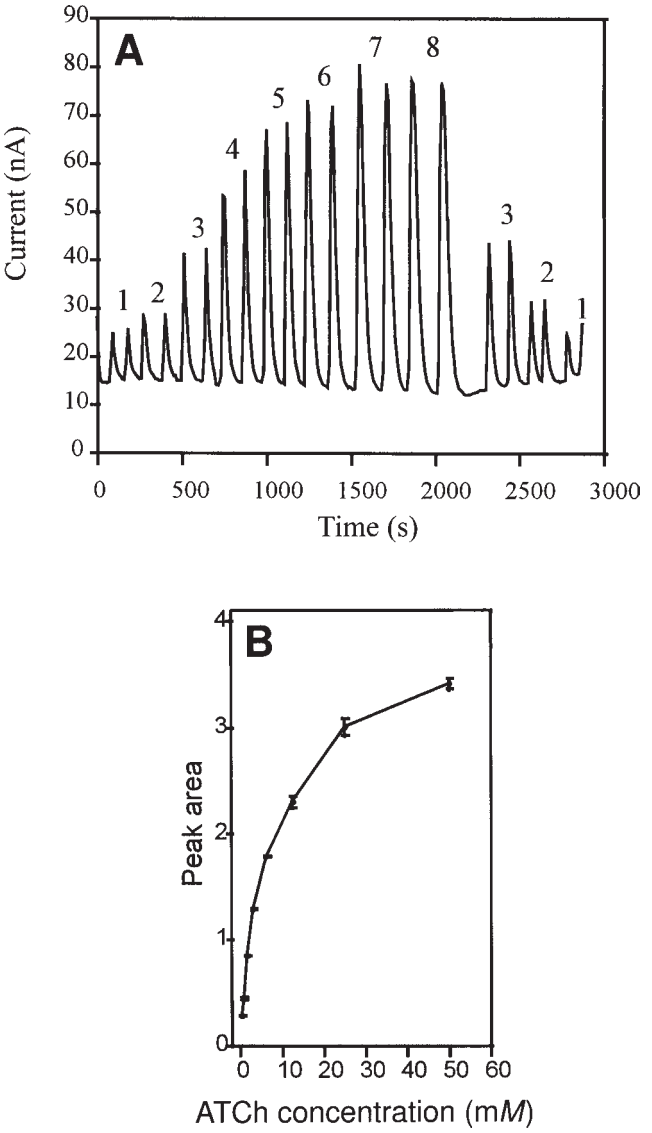


Fig. 3. **(A)** Chronoamperometric curves showing the response of AChE-based sensor to injection of 10 μL of different concentrations of ATCh. Each concentration was injected twice. 1, 0.39 m*M*; 2, 0.78 m*M*; 3, 1.58 m*M*; 4, 3.12 m*M*; 5, 6.25 m*M*; 6, 12.5 m*M*; 7, 25 m*M*; 8, 50 m*M*. **(B)** Calibration curve for ATCh.

formation rate on the inhibitor concentration. The slope of the graph is K_I, which is the inhibition coefficient *(20,21)*:

$$2.3 \times \log[100(A_t/A_0)]t = K_I[I]$$

We measured the enzyme activity before and after exposure to the inhibitor and checked various substrate concentrations. A substrate concentration of 1 m*M* and a flow rate of 100 μL/min were found to yield the

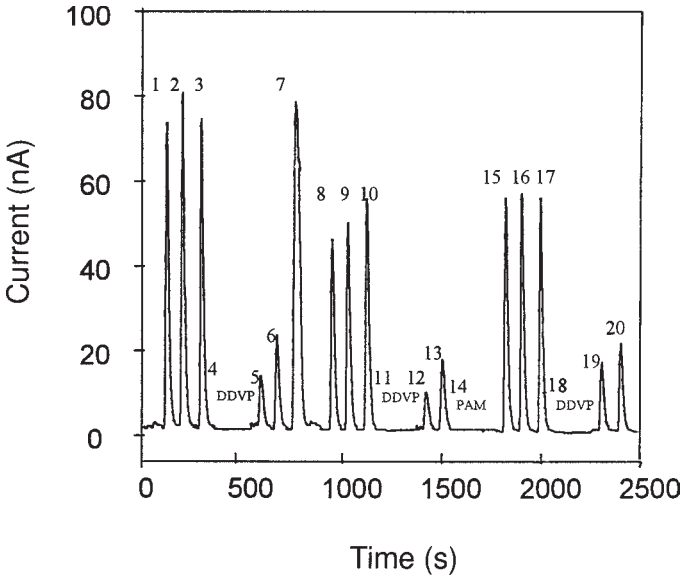


Fig. 4. Amperometric response of the AChE sensor to ATCh addition before and after exposure to DDVP and PAM. Signals 1–3, 8–10, and 15–17: addition of 1 m*M* ATCh; signals 4, 11, and 18: addition of 50 ng of DDVP; signals 5–6, 12–13, and 19–20: ATCh addition after exposure to DDVP; signal 7: addition of 10 m*M* ATCh; signal 14: addition of PAM.

highest reproducibility. Enzymatic activity could be recovered either by washing with a high concentration of the substrate or by adding 0.1 m*M* of PAM, which displaced the DDVP.

In a typical experiment, 5 μL of a 1 m*M* solution of ATCh was injected into the system and the initial enzyme activity was recorded. The exact time in seconds, τ, between the injection and the beginning of the current peak was determined. Thereafter, 5 μL of DDVP was injected and after τ s the flow rate was changed from 100 to 1 μL/min. After incubation with the inhibitor at a specific time period, the flow rate was changed again to 100 μL/min, the unbound inhibitor was washed away, and a new sample of the enzyme was injected.

Figure 4 shows the enzyme electrode response to ATCh and DDVP. The signals obtained are very sharp and reproducible. It is evident that 50 ng of DDVP decrease more than 80% of the enzyme activity. Applying a high concentration of the substrate thiocholine (5 μL of 10 m*M*) almost fully restored AChE activity (Fig. 4) *(7)*. Incubation of the enzyme with PAM had the same effect (Fig. 4) *(14,15)*.

Two main factors influenced the degree of inhibition: the quantity of inhibitor added, and the exposure time of the enzyme to the inhibitor. In another set of experiments, different amounts of DDVP (12.5–100 ng) were injected into the cell for various exposure times and the degree of inhibition was calculated. Figure 5 shows the linear correlation found between the amount of inhibitor and the logarithm of the relative activity

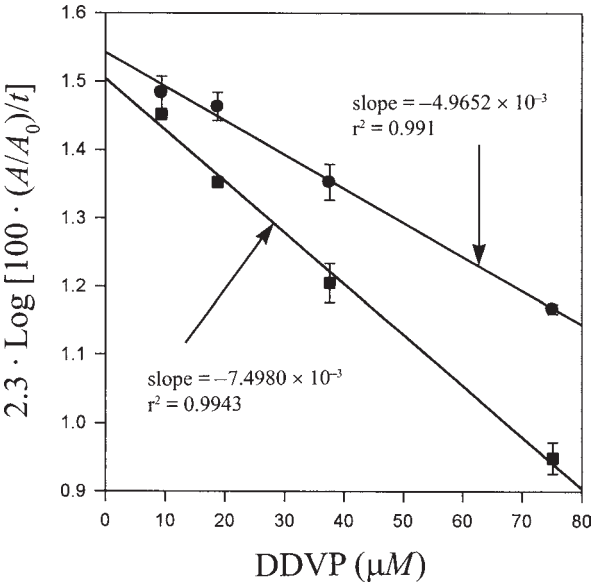


Fig. 5. Enzyme activity in response to DDVP. Logarithmic dependence of relative enzyme activity on the amount of DDVP. Exposure time = 3 min; upper curve pH = 7.3; lower curve pH = 8.0.

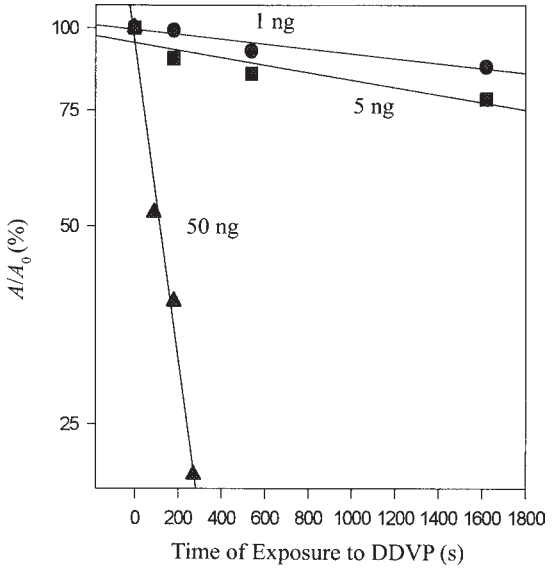


Fig. 6. Relative enzyme activity in response to various exposure times to DDVP.

(activity/initial activity). The inhibition coefficients at pH 7.3 and 8.0 were 4.96×10^3 and 7.5×10^3 M/min, respectively. These values are consistent with those reported in the literature *(20,21)*. The enzyme activity directly depended on the applied inhibitor concentration as well as on the exposure time, as shown in Fig. 6. As the inhibitor concentration increased, the rela-

tive activity of the enzyme decreased. Similarly, as the incubation time was extended, enzyme activity decreased. These results are of great importance for determining an unknown quantity of DDVP: by changing incubation time and monitoring residual enzyme activity, we could increase the concentration range of organophosphate compounds measured by the sensor.

The presented system serves as a good example for a disposable amperometric flow injection sensor that can be applicable to the identification and quantification of organophosphate compounds in the environment. The sensor is sensitive, rapid, small, and cheap. The miniature flow cell system, combined with SPEs, allows the advantage of working with low volumes of samples containing hazardous organophosphate compounds.

Quantitative Determination of Lactose

Enzymatic methods involving β-galactosidase and GOD have been described. These methods are highly specific, using immobilized rather than free enzymes together with colorimetric methods and chromogenic agents *(22)* for response detection. We have developed a sensitive electrochemical biosensor for lactose determination based on three successive enzymatic reactions:

$$\text{lactose} \xrightarrow{\beta\text{-GAL}} \text{glucose} + \text{galactose}$$

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} H_2O_2 + \text{gluconolactone}$$

$$[H_2O_2 + Fe(CN)_6]^{-4} \xrightarrow{\text{POD}} [Fe(CN)_6]^{-3} + H_2O$$

The current was generated owing to the reduction in the $[Fe(CN)_6]^{-3}$ at the electrode according to the following reaction:

$$[Fe(CN)_6]^{-3} + e^- \longrightarrow [Fe(CN)_6]^{-4}$$

Figure 7A shows the current response to injection of different concentrations of lactose. Figure 7B shows the calibration curve for lactose. A linear region was obtained between 10 and 375 μg/mL of lactose and the optimal pH was found to be 6.2.

We have detected lactose in yogurt, milk powder, pasteurized milk, and raw milk. Figure 8 shows an example of lactose detection in raw milk by the standard addition method.

Ultimately, we have examined the reliability of the biosensor and its shelf life. Lactose concentration in cow's milk was measured at other laboratories as well, using the same electrodes. The results deviated by 3% at the most. The shelf life of the biosensor was at least 5 mo. Although the activity of the electrodes had decreased with time, calibration of the electrode before use overcame this problem.

The biosensor for lactose determination comprises an attractive useful tool. It can trace a concentration of lactose of a few micromoles (about 6000 times diluted compared to pure milk), it is reproducible and stable for

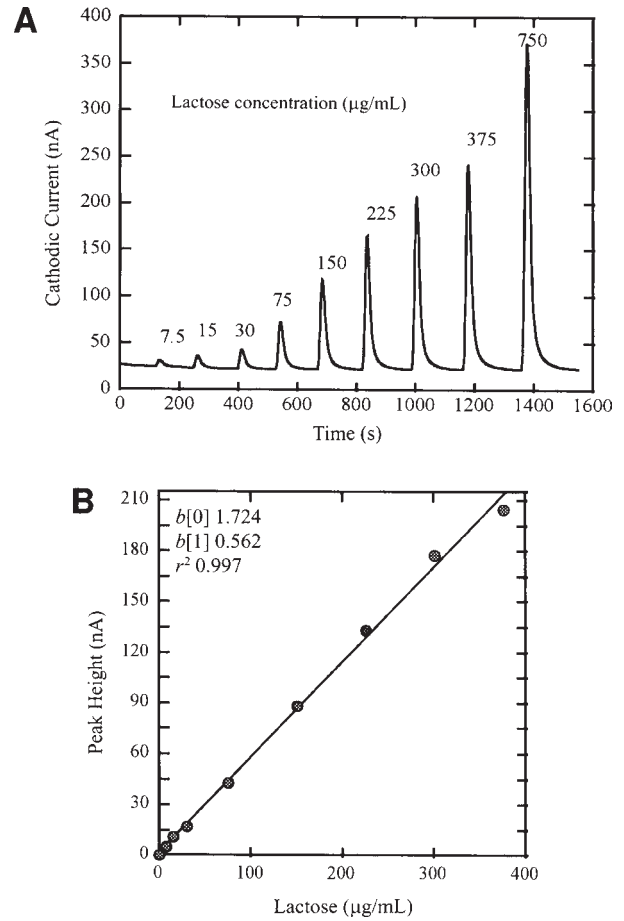


Fig. 7. Detection of lactose. **(A)** Amperometric response of the lactose sensor to additions of lactose; **(B)** calibration curve of lactose.

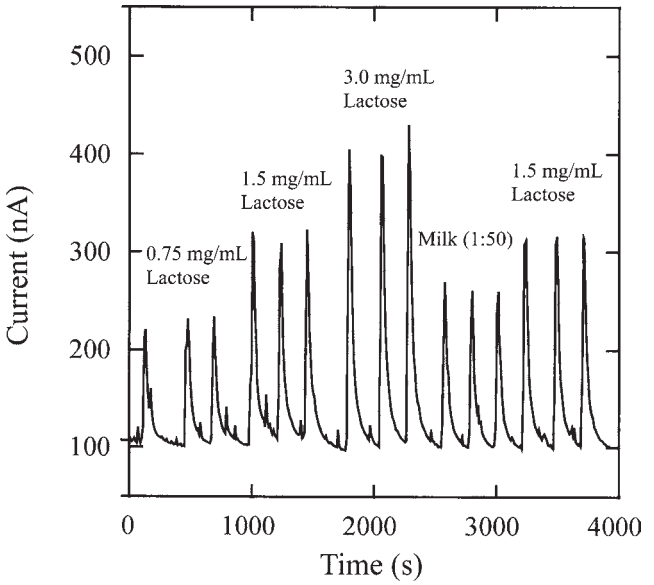


Fig. 8. Detection and quantitative determination of lactose in raw milk.

a relatively long time (at least 6 mo when stored in solution), it has a short time response, and, above all, it is based on a cheap and simple procedure and hence can be used on the spot in dairy farms.

Conclusion

We have developed a disposable amperometric flow injection sensor that, as demonstrated, can be applicable in various instances. The sensor is sensitive, rapid, reproducible, inexpensive, and compact. The miniature flow cell system, combined with SPEs ensures the continuous flux of a new substrate, therefore preventing accumulation or adsorption of products near the working electrode. Moreover, miniaturization of the biosensor offers the advantage of working with low volumes of materials and reagents that may be hazardous, such as organophosphates. Finally, because the sensor is inexpensive and simple to operate, it can be used equally well indoors and outdoors and is disposable as well, thus allowing simple and efficient measurements.

References

1. Ziegler, C. and Gopel, W. (1998), *Curr. Opin. Chem. Biol.* **2(5),** 585–591.
2. Luong, J. H., Bouvrette, P., and Male, K. B. (1997), *Trends Biotechnol.* **15(9),** 369–377.
3. Mosbach, K., ed. (1988), *Methods in Enzymology*, vol. 137, Academic, New York.
4. Turner, A. P. F., Karube, I., and Wilson, G. S., eds. (1987), *Biosensors: Fundamentals and Applications*, Oxford University Press, New York.
5. Markas, A., Gilmartin, T., and Hart, J. P. (1994), *Analyst* **119,** 2431–2437.
6. Huang, Y., Qiao, C., Williamson, M. S., and Devonshire, A. L. (1997), *Chin. J. Biotechnol.* **13,** 177–183.
7. Ashani, Y., Rothschild, N., Segall, Y., Levanon, D., and Raveh, L. (1991), *Life Sci.* **49,** 367–374.
8. Yang, M. T., Milligan, L. P., and Mathison, G. W. (1981), *Chromatography* **209,** 316–322.
9. Garrison, E. R. (1939), *J. Assoc. Off. Agric. Chem.* **33,** 489–495.
10. Davis, J. G. (1953), *Richmond's Dairy Chemistry*, C. Griffin Pubs., London, pp. 361–391.
11. Dosoretz, C., Armon, R., Starosvetzky, J., and Rothschild, N. (1996), *J. Sol-Gel Sci. Technol.* **7,** 7–11.
12. Rothschild, N., Levanon, D., Gertler, A., Sakal, E., and Henis, Y. (1990), *Proc. Annu. Meeting Israel Soc. Microbiol.*, Israel Soc. of Microbiol., ed., p. 23.
13. Huang, Y., Qiao, C., Williamson, M. S., and Devonshire, A. L. (1997), *Chin. J. Biotechnol.* **13(3),** 177–183.
14. Guilbault, G. G., Hock, B., and Schmidt, R. (1992), *Biosens. Bioelectron.* **7,** 411–419.
15. Marty, J. L., Mionetto, N., Lacrote, S., and Barcelo, D. (1995), *Anal. Chim. Acta* **311,** 265–271.
16. Kumaran, S. and Tran-Minh, C. (1992), *Anal. Biochem.* **200,** 187–194.
17. Ivnitskii, D. M. and Rishpon, J. (1993), *Analytica Chimica Acta* **282,** 517–525.
18. Gunter, A. and Bilitewski, U. (1995), *Anal. Chim. Acta* **281,** 3–11.
19. Rippeth, J. J., Gibson, T. D., Hart, J. P., Hartley, I. C., and Nelson, G. (1997), *Analyst* **122,** 1425–1429.
20. Alfthan, K., Kenttamma, H., and Zukale, T. (1989), *Anal. Chim. Acta* **217,** 43–51.
21. Skladal, P. and Mascini, M. (1992), *Biosens. Bioelectron.* **7,** 335–343.
22. Dyer, N., Stoute, V. A., Gershwin, D., and Ngo, T. T. (1991), *Analyt. Biochem.* **194,** 16–24.