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RESEARCH ARTICLE

Nanobioprobe construction of acetylcholinesterase by Using of Optical Fiber

Khederzadeh, Masumeh, Molaei rad, Ahmad
Faculty of Science, and Research Campus, Islamic Azad University, Tehran, Iran

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Corresponding Author

Khederzadeh, Masumeh

Abstract

The issue of organophosphates and their usage and effects on environment has being becoming an important issue in academia. In recent years, several methods have been developed by researchers to measure paraoxon organophosphere on a global scale. In this paper, we investigate this problem and introduce a novel Nano fiber-optic bioprobe system to detect paraoxon as an enzyme inhibitor. The fiber-optic enzyme biosensors are included an acetylcholinesterase (AChE)-immobilized by cross-linking glutaraldehyde on silica optic fiber. The measurement of reducing of products 5,5'-Dithio-bis(2-nitrobenzoic acid) using paraoxon on AChE is used as a technique to sense scheme. The environment temperature and the response time to steady signal of sensor were 25 °C and 5 min., respectively. Also, the substrate affinity was constant and K_m for AChE in control was observed as optic fiber 0.43µM. Moreover, the V_{max} for AChE immobilization on Optic Fiber was constant at 0.27ΔA/µgprotein/min. Experimental results indicated we could detect absorbance 5,5'-Dithio-bis(2-nitrobenzoic acid) at 412 nm, Paraoxn value. Moreover, we could detect paraoxon at very low-quantity sample. We could detect the detection method from 10^{-9} Molar using our proposed biosensor.

INTRODUCTION

Acetylcholinesterase's biological terminates impulse transmissions at cholinergic synapses within nervous systems of insects and mammals using rapid hydrolysis of acetylcholine [1]. This material may block an active part of a nerve enzyme by using nucleophilic attack [2]. The principles of the detection by biosensors are usually based on inhibition by organophosphorus compounds of AChE. AChE is an enzyme with narrow specificity for acetylcholine and related compounds in nerve tissue [3].

In the recent years, a detection of organophosphates has been becoming an interest topic in academia due to its extensive usage and effects on the environment. Unfortunately, organophosphates are too toxic and only little portion of them can cause a lot of hazardous diseases for persons [4]. Convention organophosphates techniques such as gas-layer chromatography [5] and thin-layer chromatography [6]are used as analytical methods with high accuracy. Nevertheless, these methods have several noticeable disadvantages; therefore, these methods are not suitable in practical terms. For example, these methods are too expensive and require highly qualified technicians. In addition, some methods may consume much time. Biosensors and bioprobes devices are novel opportunities to decrease complexity and cost of organophosphates detection techniques [7]. Biosensor and bioprobes devices have several advantages. For instance, these novel techniques are so inexpensive, sensitive and portable. In addition, they are easy to use and capable to produce reliable analytical information [8]. Also, an electric field cannot affect fiber-optic sensors, easily [9]. These devices can be used in continuous assessment of organophosphates [10].

Due to these abilities and advantages, biosensor devices are noticed by researchers. In [5-7] authors established biosensors to detect an organophosphorus compounds based on inhibition of AchE by organophosphorus compounds. In [11, 12] researchers utilized AchF modified amperometricn and developed biosensors for organophosphorus based on the AChE inhibition test. Furthermore, biosensors can be used to measure PH changes during enzyme's reactions which these enzymes are related to density of organophosphorus [13]. Fluorescent was studied in [14] and colorimetric in [14-16]. Incorporation of porins within the liposome membrane [17] and optical [18] facilitate substrate partitioning into the liposome [19].

In the present study, we know the adsorption of organophosphor compounds, such as paraoxon detection using AchE immobilize on the nanooptical fiber surfaces is a well-known phenomenon [20]. In addition, optical fiber improves stabilization of enzymes, therefore, biosensors with higher operational and storage stabilities are provided. Hence, based on the idea of paroaxon selective concentrations, we develop immobilized enzyme on fiber-optical biosensors system to detect paraoxon[3].

The rest of paper as follows. In section 2, we describe materials and methods. Results and discussions are explained in section 3. In addition, we describe conclusion in section 4.

1- Materials and Methods

2-1- Reagent

AChE (E.C. 3.1.1.7), acetylcholine chloride (Acthch), Paraoxon (diethyl-p-nitrophenyl phosphate), glutaraldehyde 25%, , 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), Glycidoxy propyl trimetyl silanat (GOPS), L-α phosphatidylchaline, pyrene, Sigma Chemical Company which all above materials were produced by St. Louis, MO, USA. Selica optic fiber, phosphoric acid, phosphate buffer 1M (pH 8.0). All samples were analyzed at room temperature.

2-2- Nanofiber fabrication

We used heat-and-pull method to fabricate Nano fiber in our experimental procedure. In this method, fiber is pulled at both sides to decrease its diameter. We used a special fiber-pulling device to pull fiber which is shown in figure 1 (SutterInstrumentsP-2000, Novato,CA). The fiber is fixed into the fiber-pulling device. The laser heating source is focused onto the median point of fiber. This method uses CO2 laser to utilize local heating of silica fiber. The resulting tip shapes strictly related on experimental parameters such as environment temperature and time of the procedure. Based on method that was introduced by Hoffman [21], the optical fibers is pulled to the breaking point, therefore, we could produce two Nanofibers using this method.

2-3- **Coating of Nanofiber**

In the next step, we need to cover the tapered side walls of optical fiber. This step is an important step in the Nanosensor fabrication process. We used a thin layer of silver, aluminum or gold with diameter between 100- 200 nanometer for coating the tapered side walls of the optical fiber. Cooke vacuum Evaporator system (South Norwalk, -6 CT) can be used to accomplish this step. The coating procedure helps distal end of the fiber to be free from silver for subsequent derivatization and allows covalent immobilization of biological sensing elements to exposed silica nanotip. It is worth to notice, the angle between fiber axis and the evaporation direction is approximately 45 degrees [22].

2-4- **Immobilization of AchE on optic fiber**

Now, after coating of Nanofiber, we want to immobilization of AChE on optic fiber. To achieve this purpose, we need to derivatization of the Nano tips. Our goal is to facilitate the covalent immobilization of biological sensing molecules. According to the following immobilization procedure, the AChE enzymes were cross-linked with controlled pore glasses.

Silica surfaces were notorious for physical adsorption of proteins. However, we used silica because electrical and magnetic fields do not have any effects on it. In addition, silica is flexible and has ability to transmit and receive optic signals. Acetylcholinesterases were used to a glutaraldehyde. By using the method that explained in [23], both acetylcholinesterases can be immobilized onto the optical Nanofibers. Batch of 10 optic fiber probes were used in immobilized. Fiber probes needed to be cleaned; therefore, we cleaned it by deionized water and submerged them in the concentrated H_2SO_4 for 30 minutes. Then, we rinsed them and boiled them in deionized water for 8-10 minutes.

Immersion in freshly prepared 1% (v/v) solution of DETA in 1mM ascent acid for 20 minutes at room temperature is employed to silanization of fibers. For eliminating extra DETA, the DETA modified fiber probes were rinsed with deionized water. Then, the silanized fiber probes were dried under N_2 and stabilized with heat at 120°C for 5 minutes. For utilizing a bifunctional cross-linker and glutaraldehyde, we carried out conjugation of the tips. Based on [24], we kept silanized probes at 12.5 %(v/v) glutaraldehyde solution with 0.1 M potassium phosphate buffer solution (PBS) at pH 8 for 14 hours at room temperature.

2-5- Optical sensing system

Due to the small sampling volume probes in Nanosensors, the amount of target enzyme in the excitation volume was too small. Therefore, we needed to use a sensitive optical spectroscopic technique. For example, fluorescence is proper technique for an analysis. Moreover, we needed to design and employ a sensitive measurement and detection system. We tested results of immobilization of AChE for paraoxon detection. An inhibition of AChE was attributed to an acylation of a serine-OH in the active site of an enzyme using these compounds. Based on [25], the simplest optical bioprobes utilize absorbance measurements to determine any variations in the concentration of analytes that absorb a given light wavelength.

2-6- **Paraoxon concentration measurements**

We know, probably, paraoxon is too dangerous and have been responsible for many accidental poisoning and dead than other organophosphous insecticides. Hence, in our research, we wanted to measure paraoxon concentration. Baseline with different concentrations (10-6 - 5×10-9M) of paraoxon solution in DMSO were obtained by using kinetic measurement with immobilized enzyme. We first recorded baseline and then introduced it into the measurement chamber and at the end, determined kinetic responses. It is worth to notice that we used initial kinetic for calculating the reaction rate. The authors in [26] emphasized that reaction rate indicate linear correlation between substrate concentration and adsorption.

2- Results and discussions

In this section, we explain experimental results and discuss about them. Hence, we divide this section into three parts including fabrication Nano fiber, enzyme binding and directs immobilization of AchE inhibition.

3-1- **Fabrication Nano fiber**

Construction of Nanometer-sized tips on optical fibers is foundation of optical-fiber-based Nanosensors. Different tip diameters, taper angles and fiber-surface smoothness are required for different applications and environments. Pulling instruments such as micropipette puller are the conventional way to prepare Nanometer tripped optical fibers. Therefore, in this research, the diameter of fiber tips were very reproducible from fiber to fiber by establishing different heating temperatures and tension that applied to fibers. According on Atomic Force Microscopy (AFM) images of 10 fibers that illustrate in figure 2, the diameter was changed between 50-200 nm. This smooth shape and its small size allow us to insert individual fiber into bioprobe.

3-2- Enzyme binding

Bradford method was established by us to determine the amount of bound AChE. We first immobilized AChE on silica optic fiber and then calculated AChE binding on optic fiber. After calculating this binding, we investigated the effects of substrate acetylcholine chloride concentration on kinetic of reaction catalyzed by immobilized AChE. We studied acetylcholine chloride in the presence of Buffer Phosphate Sodium at temperature 25 ◦C and pH 8.0. Based on results that are illustrated in Table 1, we could calculate Michaelis constant (Km) and the maximum reaction rate (V_{max}) together with the corresponding value of the free AChE. The value of Km of free enzyme was lower than the values of Km that calculated for AChE immobilization on optic fiber.

3-3- D**irects immobilization of AchE inhibition**

Recently, because of high intensive work in the field of electrochemical bioprobs, researchers have been interesting to immobilization of AChE. Immobilized AChE is used to detect organophosphorous. Calibration curves for paraoxon determination are based on decreasing AChE activity in the presence of paraoxon. Paraoxon causes irreversible inhibitory of enzyme. Concentration ranges of paraoxon were tested from 10^{-6} M to 5×10^{-9} M to survey their effects on enzyme activity at different incubations. According to results in [9], until reaching constant level in 3-10 minutes, the degree of enzyme inhibition increases when the incubation period increases. Therefore, we considered 5 minutes as the optimum time of inhibition. The "calibration plots" of inhibition degree vs. paraoxon concentration is shown in Fig. 4. A first-order reaction controlled the range of inhibition. To obtain this figure, we consider the slope of linear range is the steepest of all concentrations. We could reach a percentage inhibition of 73% for paraoxon 0.5µM.

Percentage of inhibition is linearly related to paraoxon concentration in the range of 10^{-9} nM to 10^{-6}M. Therefore, the percentage of inhibition increases by increasing paraoxon concentration. AChE immobilized on optic fiber exhibited the greatest sensitivity toward the paraoxon. We achieved non-linear relationship between paraoxon concentration and percentage of inhibition when paraoxon concentration increased more than this mentioned range. Figure 5 indicates absorption vs. time for different paraoxon concentrations. Based on this figure we observed there is a linear relationship between absorption and time for the immobilized enzyme from 10^{-6}M to 5×10^{-9}M. These results indicate if we want to construct paraoxon detection, discussed immobilized system is potential system for construction bioprobes. Our detection limitation was 10^{-9} M and in some cases, this value was better than values of detection limitation that obtained by other researchers. A common mode for application of bioprobe for AChE-based recognition requires a long preincubation procedure. However, this method provided measurement of concentration of paraoxon in presence of DMSO solution with a detection limit as low as 10^{-9}M for us. This observation is shown in figure 6. It is so necessary for bioprobe to be simple for practical terms. In addition, for practical application, bioprobes should have low detection time, short response time, and storage stability and require very less amount of sample. Moreover, it is very important for bioprobe's disposable biocomponent to be cost-effective. We used bioprobe for detection of organophosphate paraoxon and compared detection limit of bioprobes with available literature on either enzyme or optic bioprobe, in this study.

Our results confirmed that such as free AChE, the immobilized AChE on optical fiber has high sensitivity to paraoxon. Nevertheless, free AChE is more eager than immobilized AChE for combination with paraoxon. This research indicated the activity of immobilized AChE in presence of paraoxon is less than free AChE. Based on our measurements, immobilized AChE activity is only 76 percent of free AChE activity. We have described optic fiber bioprobe to determine paraoxon. The advantages of detection of paraoxon using immobilized AChE with optic fiber are simple and mono-step. Also, we can measure of very low quantity of sample, directly. Disposable enzyme was cost-effective for paraoxon detection. This method is suitable for analyzing performance of paraoxon. We selected range (10^{-6} - 5×10^{-9}M) paraoxon in our study and obtained 10^{-9}M as the lowest detection limit.

3- Conclusion

We investigated effects of paraoxon on immobilized enzyme on the optical fiber in this paper. Paraoxan has powerful inhibitory effect on immobilized enzyme. The activity of enzyme is investigated in the presence of different concentrations of paraoxon. We could detect 10^{-9}M as the lowest detection limit. Hence, we can use immobilized enzyme on the optical fiber as bioprobe in the optical biosensors. These bioprobes can be used to detect low-concentration of paraoxon. The advantage of our method is that we can use bioprobe for several times. This helps us to save money. In addition, our method has high sensitivity.

Legends

Figure 1, the device is used in the heat-and-pull method.

Figure 2, image of Nanofiber that obtained by optical microscope. The tip diameter of the tip is approximately 200 nm.

Figure 3, Atomic Force Microscopy (AFM) image of Nanofiber. The tip diameter of fiber is approximately 50 nm.

Figure 4, spectrophotometric spectra of assay of immobilized AchE on the optical fiber in the presence of A: 0.1 M sodium phosphate buffer; pH 8.0; 50µL AchE 0.001mM; 100µL of acetylthiocholine 0. 5 µM; at 412nm. B: 0.1 M sodium phosphate buffer; pH 8.0; 50µL AchE 0.001mM; 100µL of paraoxon 0. 5 µM; at 412nm.

Figure 5, spectrophotometric spectra of assay of immobilized AchE on the optical fiber in the presence of 0.1 M sodium phosphate buffer; pH 8.0; 50µL AchE 0.001mM; 100µL of A: 100µL of acetylthiocholine 0. 5 µM; B: Paraoxon 10-9 M; C: Paraoxon 10-8 M; D: Paraoxon 10-7M; E: Paraoxon 0.5 µM; at 412nm..

Figure 6, Calibration curve of inhibition of AchE in the presence of Paraoxon concentrations.

Table 1, Comparison of kinetic parameters of immobilized and solution of AchE.

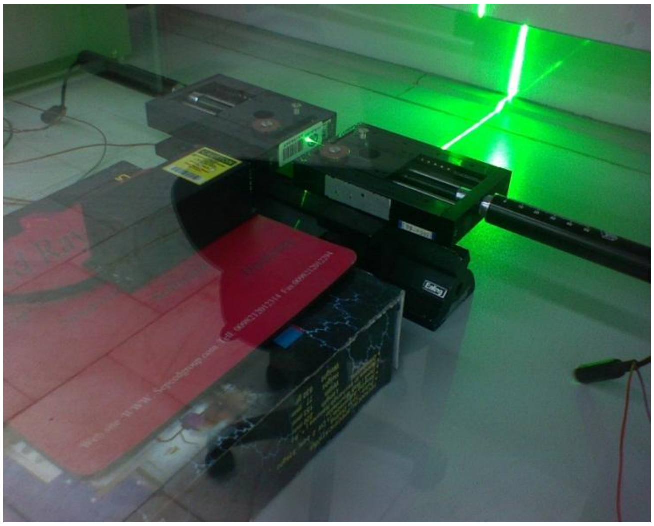

Fig.1.

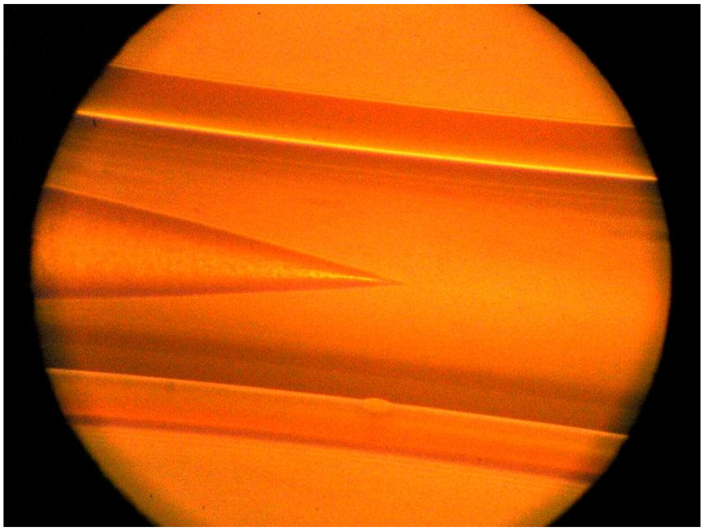

Fig.2.

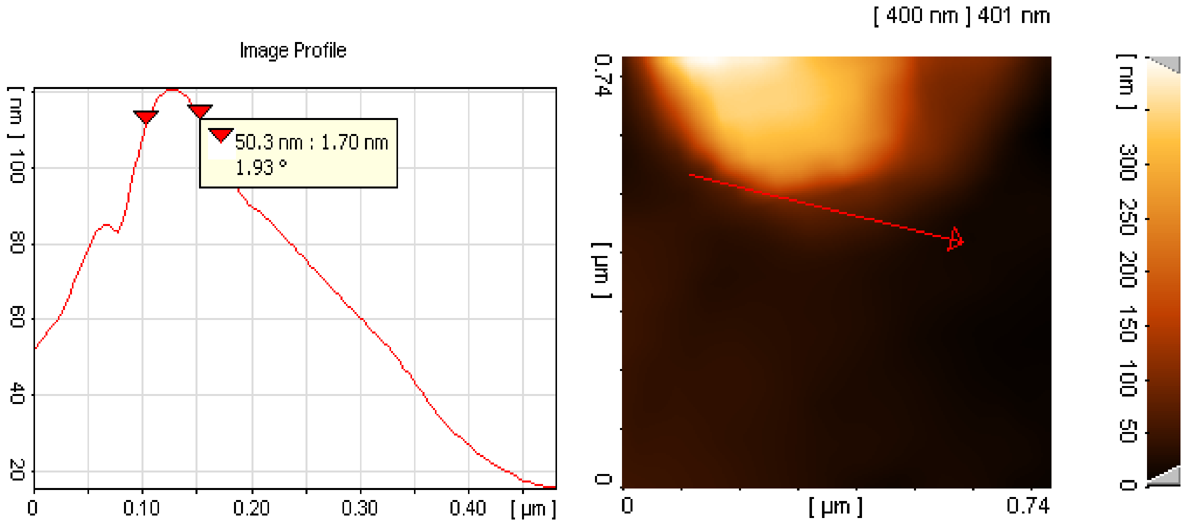
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Fig.3.

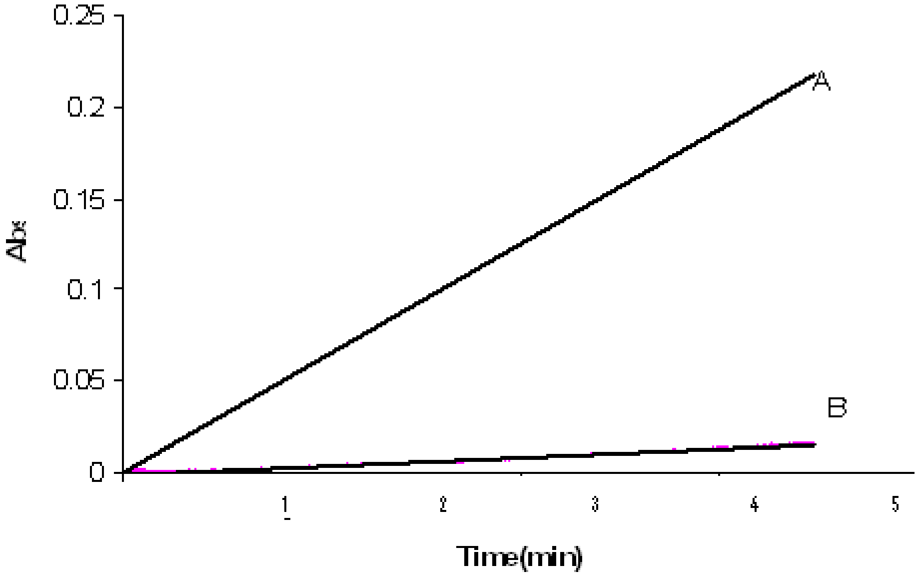
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Fig.4.

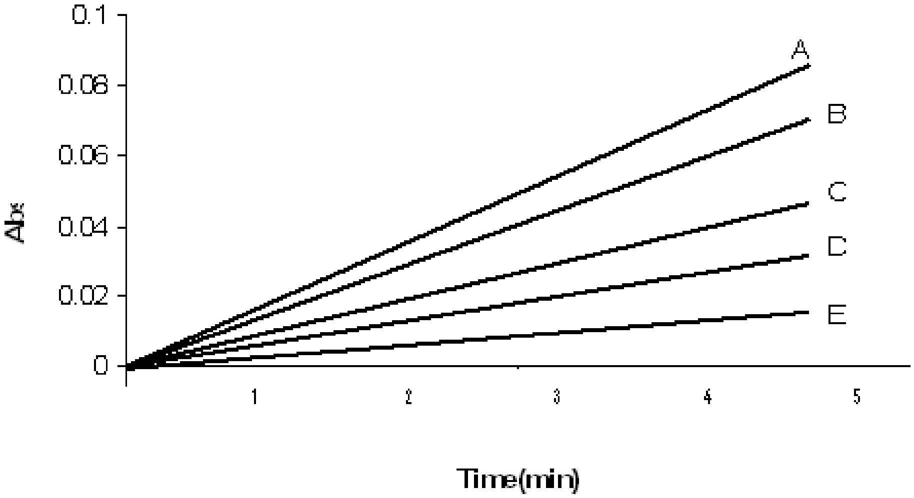

Fig.5.

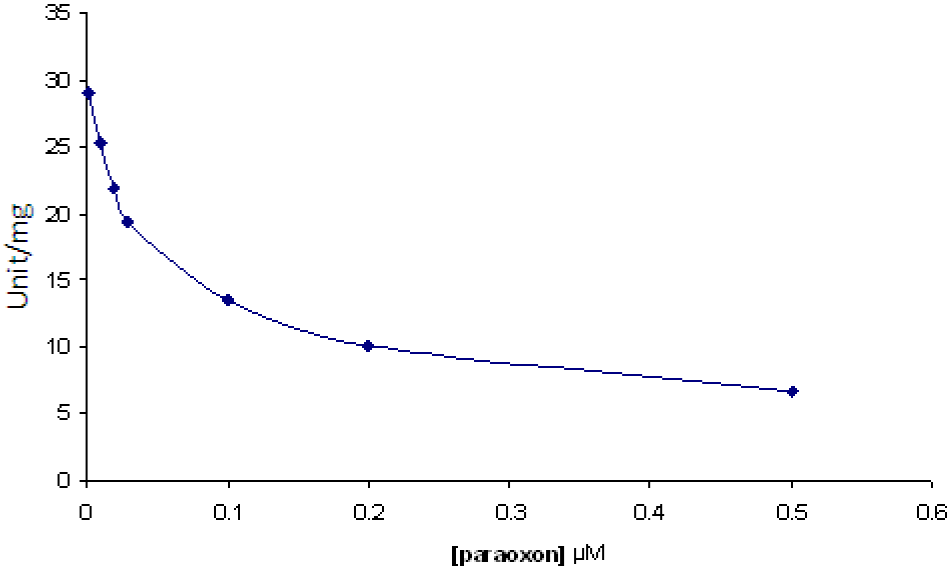

Fig.6.

Table 1:

AchE	Km	v_{max}
control	0.27	0.34
Immobilized Enzyme	0.43	0.27

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