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ENZYMIC ANALYSIS OF ORGANOPHOSPHATE INSECTICIDES USING AN ENZYME THERMISTOR

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Quantitative analysis of water soluble insecticides using an enzyme thermistor was examined for organophosphates. A procedure based on an insecticide hydrolyzing enzyme was found suitable for continuous determination of insecticides at concentrations down to 30 μM. A more sensitive procedure, < 3.4 μM, suitable for discontinuous monitoring was developed using reversible immobilization of acetylcholine esterase.

INTRODUCTION

Environmental analysis of synthetic chemicals has grown in importance in parallel with awareness of the dangers of a polluted environment. Most analytical systems currently used in environmental analysis consist of conventional analytical systems that have been slightly modified to fulfill the newly arising specific demands.

During the last few years, the design of analytical systems based on enzymes or microorganisms have begun. Thus there are reports concerning detection and analysis of air-borne pesticides (1) and analysis of cyanide (2) and heavy metals (3) using biologically based procedures. There are also less specific systems based on immobilized whole cells (4–6). These cells, with their great diversity of enzymic reactions, respond to a broad spectrum of chemicals present in the medium. Such immobilized cells have been used in a convenient BOD-sensor for assaying biological oxygen demand, a value that is related to the total content of organic material in the waste water sample (5). Other similar systems have been developed that are sensitive to a broad spectrum of inhibitors and can thus measure the integrated effect of pollutants in water (6). The present paper deals with the enzymic analysis of

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organophosphate insecticides by detecting the heat released during enzymic hydrolysis of the water soluble insecticide by an immobilized enzyme in a thermistor.

MATERIALS AND METHODS

A parathion degrading enzyme was isolated from a mixed bacterial culture, which was previously adapted to parathion metabolism (7). This culture consisted of at least nine isolates, five of which were species of fluorescent pseudomonads; the remaining four isolates were classified as *Brevibacterium*, *Azotomonas*, and *Xanthomonas* sp., and an unidentified bacterium. The mixed culture was grown in a Braun-Melsungen AG Biostat 5 fermentor (4-l working volume) for 7 days before harvesting (8).

This crude enzyme preparation was able to hydrolyze not only parathion (*o-o*-diethyl-*o-p*-nitrophenyl phosphoric acid), but a wide range of other organophosphate pesticides, which included methyl-parathion, parathion, dursban, diazinon, triazophos, cyanophos, EPN, fenitrothion, and DDVP (8). The enzyme preparation was obtained by harvesting the cells by centrifugation (10,000 *g*, 15 min), resuspending the pellet in 10 mM phosphate buffer, pH 7.2, and then sonicating the cells for 12 s/ml. Specific activity of this crude enzyme preparation ranged from 0.6 to 5.0 μmol/parathion hydrolyzed/min/mg protein.

All pesticides used in this experiment were of greater than 95% purity, and were obtained from Bayer AG, W. Germany, E.I. DuPont de Nemours, Wilmington, Del., or Hoechst, W. Germany. Acetylcholine esterase from horse serum (EC 3.1.1.7) and butyrylcholine were purchased from Sigma (St. Louis, Mo.). Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Controlled pore glass CPG 10-70, mesh 80/120, pore diameter 729 Å was obtained from BDH, Poole, England.

Enzyme Thermistor Equipment

The enzyme thermistor is a kind of simple flow calorimeter containing a small insulated glass column (total volume used, 0.5 ml) filled with immobilized enzyme (9). A continuous flow of preequilibrated buffer passes through the column in an upward direction. A thermistor is inserted in the top of the column (Veco 41A28, temperature coefficient, −4.4%), and the device is coupled to a Wheatstone bridge, an amplifier, and a strip chart recorder.

For the studies discussed in this report, two different devices have been used. For direct enzymatic assay, a split-flow enzyme thermistor was used (10). Here the flow is split between two columns, one containing active

enzyme and the other, a reference column, filled with inactive enzyme. This device is well suited for analysis of media with unknown compositions. A more simple enzyme thermistor device, containing only one column (11, 12), was used in inhibition studies where the risk of heat fluctuations from unspecific reactions was very low.

Immobilization Procedure

Insecticide Metabolizing Enzyme. Controlled pore glass was used as support and immobilization to γ-aminopropylsilanized glass was performed using glutaraldehyde as coupling agent (13).

Horse Serum Acetylcholine Esterase. This enzyme was reversibly immobilized on Concanavalin A-Sepharose. The enzyme was dissolved in perfusion buffer and introduced into the flow of an enzyme thermistor unit that contained Con A-Sepharose. The enzyme, which is a glucoprotein, was bound via its carbohydrate residue to the immobilized lectin, Concanavalin A. The enzyme could be desorbed from the column by a pulse of 0.2 M glycine-HCl, pH 2.2 (14).

Assay Procedure

The Organophosphate Insecticide Hydrolyzing Enzyme. The insecticide to be analyzed was introduced into the flow (flow rate 0.76 ml per min per column) as a pulse. The insecticide was first dissolved in methanol as a 1% stock solution, and small volumes were then dissolved in the perfusion buffer (10 mM Tris-HCl, pH 8.9, containing 0.1% Triton X-100). The heat liberated during the hydrolysis reaction was registered as an increase in temperature of the solute passing the thermistor.

Horse Serum Acetylcholine Esterase. The activity of the immobilized enzyme was first assayed with 10 mM butyrylcholine as the substrate; the buffer used was 0.1 M Tris-HCl, pH 7.0, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Then a 5–10-min pulse of the pesticide solution was passed through the column. This was followed by a new pulse of 10 mM butyrylcholine. The degree of inhibition was expressed as the ratio between the decrease in peak height after exposure of the enzyme to the pesticide and the value obtained before exposure to the pesticide.

An alternative assay procedure used in inhibition studies was to add a certain amount of enzyme to the insecticide-containing water and after a fixed period of time adsorb the enzyme to the lectin column. After the assay, the inhibited enzyme preparation was eluted from the column by a pulse of 0.2 M glycine-HCl, pH 2.2.

RESULTS AND DISCUSSION

Two quite different approaches were used for the analysis of the examined insecticides. The pesticide solution was passed through the column containing the immobilized bacterial enzyme system for 10 min, the plateau level was measured, and the values obtained were plotted against the substrate concentration. The standard curve for parathion analysis (Fig. 1) indicates that parathion can be accurately detected down to approximately 10 ppm (34 μM). The immobilized enzyme preparation was also tested against several other organophosphate insecticides, and the relative heat responses, i.e., the practical analytical responses under the same conditions, are listed in Table 1. The hydrolysis reaction of all insecticides tested proved exothermic. As in previous work, enzymic hydrolysis was carried out in tris-HCl buffer to increase the signals obtained, utilizing the additional heat of protonization due to interaction of the formed protons with tris molecules. From earlier reports, it was known that the pH profile of the enzyme toward the various insecticides varied, with a pH optimum in the region 8.5–9.5. The shape of the profile is also different for different substrates (8). Thus it should be possible to increase the sensitivity further by an additional factor of 2 if the procedure for each specific insecticide is optimized.

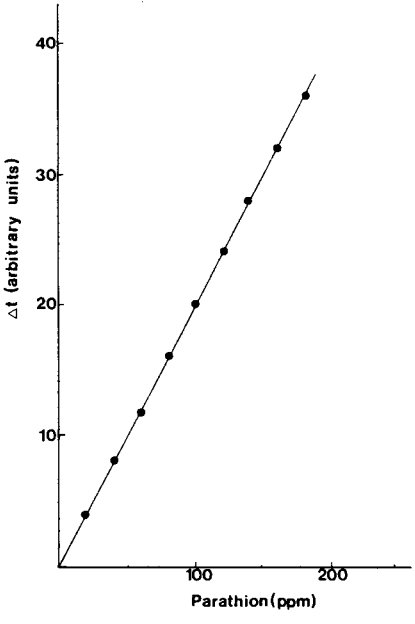

FIG. 1. Calibration curve for temperature responses at different parathion concentrations when administered to an enzyme thermistor unit containing immobilized pesticide hydrolyzing enzymes. Parathion was dissolved in 10 mM tris-HCl buffer, pH 8.9, containing 0.1% Triton X-100, and was introduced as 10-min pulses at a flow of 0.76 ml per min.

TABLE 1. **Relative Heat Responses from the Hydrolysis of Some Organophosphate Insecticides by Immobilized Bacterial Enzyme**[a]

Insecticide	Rel. heat response (%)	pH Optimum for hydrolysis (ref. 8)
Parathion	100	9.0
Cyanophos	80	9.5
Diazinon	22	9.0
Dursban	75	9.0
EPN	22	9.0
Fenitrothion	45	8.5
Methyl-parathion	70	9.0

[a]The concentration of the insecticides was 100 ppm in 10 mM tris-HCl buffer, pH 8.9, containing 0.1% Triton X-100. The heat response for the hydrolysis of parathion was set at 100%.

The second analytical approach was to take advantage of the powerful inhibition of organophosphate insecticides on certain enzymes. Acetylcholine esterase was used in these studies because it is known to be one of the *in vivo* target sites of these insecticides. The enzyme also has a very high turnover number, and this is an advantage when the degree of enzyme inhibition is to be measured. It is known from earlier studies on enzyme electrodes (15) that the sensitivity of an enzyme analytical system for inhibitors is optimal only when small amounts of enzyme are present. This, however, is contradictory to the tendency of trying to increase the operational stability of the enzyme system by immobilizing a large amount of the enzyme. In a previous report this problem was solved by immobilizing the enzyme onto pads that could be replaced after a certain time or after a certain degree of inhibition had occurred (1).

For the analysis of parathion, usually 1 U of acetylcholine esterase was immobilized on the column. Here, a reversible immobilization approach was used as described earlier (14, 16). The enzyme, a glucoprotein, was adsorbed to Concanavalin A-Sepharose, and its activity was measured prior to and after exposure to insecticides. The reaction studied was the hydrolysis of an ester with subsequent protonization of the buffer (17). The buffer used was tris-HCl, since it has been shown that the protonization heat of this buffer is far greater than that of phosphate buffer. The enzymatic hydrolysis of the butyrylcholine ester liberates almost no energy; thus the heat measured is mainly the heat of protonization of the buffer.

The results obtained when assaying the degree of inhibition of horse serum acetylcholine esterase are illustrated in Fig. 2. Less than 1 ppm

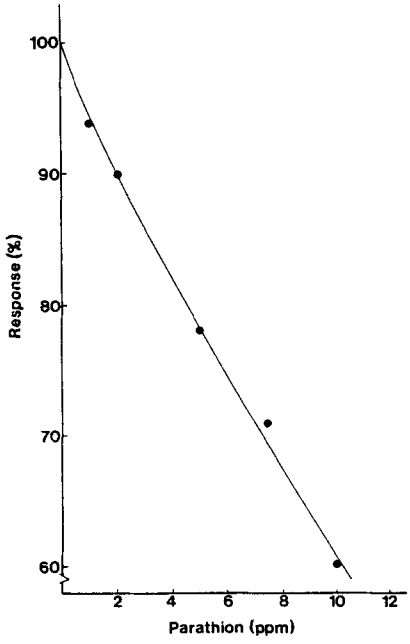

FIG. 2. Response of the enzyme thermistor containing 1 U of immobilized acetylcholine esterase to 1-min pulses of 10 mM butyrylcholine after exposure of the bed to a 10 min pulse of parathion containing buffer. (The response obtained when parathion concentration was zero was set at 100%.) Flow rate was 1.0 ml per min.

(3.4 μM) can be measured, and by increasing the time of exposure of the enzyme to the insecticide, an even higher sensitivity can be obtained. To ensure that such a procedure is reproducible after several successive adsorption-desorption steps, a standard amount of enzyme was repeatedly added to the column, and the amount bound to the column was assayed each time. Furthermore, it was shown that dissociation of the Concanavalin A-glucoprotein complex was complete after the washing step. In all these measurements, 10 mM butyrylcholine was used as substrate to ensure an excess of substrate, i.e., responses in direct proportion to the amount of enzyme bound. Figure 3 shows the results obtained on successive additions of small amounts of enzyme with no intermittent washing steps. The linear relationship indicates that the capacity of the column is good, that a small decrease in enzyme activity is easily measured, and that the reactor is limited by enzyme concentration, not substrate concentration.

The methods discussed in this paper illustrate some of the key questions in enzymatic analysis of inhibitors. Is it advantageous to use an enzyme catalyzing the transformation of the inhibitor (i.e., treating it as a substrate) or should one instead measure the degree of inhibition of a certain enzyme reaction? The answer to this question depends on the application. If a continuous quantitative analysis is needed, then the former approach is best. However, if discrete samples are to be analyzed, the second method may

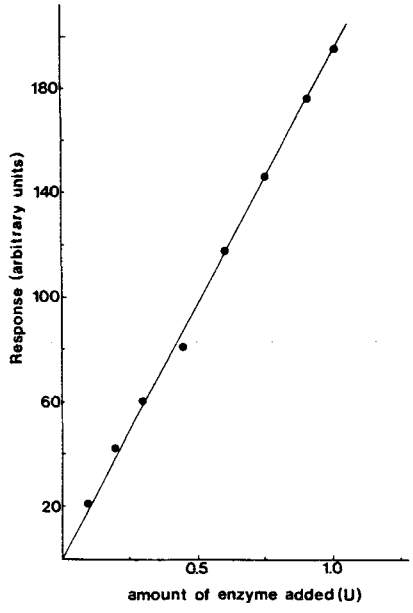

FIG. 3. Amplitude of the heat signals obtained on passing 1-min pulses of 10 mM butyrylcholine at a flow rate of 1.0 ml per min through the enzyme thermistor, which contained different amounts of immobilized acetylcholine esterase.

offer certain advantages. In the first case, the heat of the catalytic reaction is directly measured and leads to lower and lower signals as the substrate concentration decreases, whereas in the second case the signal increases in amplitude as the inhibitor concentration decreases. In the enzyme inhibition procedure each inhibitor molecule, in theory, inactivates one enzyme molecule (and each active enzyme molecule catalyzes the conversion of many substrate molecules per unit time), leading to an amplified decrease in the heat signal registered with substrate addition. In direct conversion reactions no such amplification takes place.

Relatively high levels of pesticides were used (1–10 μg/ml) in this exploratory research. However, in practical analytical applications, pesticide concentrations are expected to be below 1 μg/ml. Therefore, extreme sensitivity is needed for detection of pesticides in blood, surface waters, waste waters from pesticide production plants, etc. The enzyme inhibition procedure is the most promising of the two systems examined but still needs approximately a 10-fold increase in sensitivity.

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