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DETERMINATION OF PARAOXON BY COMBINED BOVINE LIVER ESTERASE INHIBITION AND GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A combined gas–liquid chromatography and enzyme procedure is described for the determination of organophosphorus compounds that show anticarboxylesterase activity *in vitro*. The method is based on the enzyme hydrolysis of 1-naphthyl acetate and on the determination of both the released 1-naphthol and the unhydrolyzed 1-naphthyl acetate by gas–liquid chromatography. With bovine liver as the source of enzyme the detection limit for paraoxon was 13 ppb.

INTRODUCTION

The liver esterase, sometimes referred to as "non-specific esterase" or "aliesterase", catalyzes the hydrolysis of lower fatty acid esters. A variety of assay methods are available: titrimetric[1], manometric[2] and colorimetric[3–5]. This paper describes an assay method for the determination of esterase activity in which one of the hydrolysis products (1-naphthol) and the remaining unhydrolyzed substrate (1-naphthyl acetate) are determined simultaneously by gas–liquid chromatography.

Most organophosphorus insecticides inhibit the esterase activity[6–13] and the inhibition level should be proportional to the amount of insecticides present. The proposed assay method provides a more reliable determination of organophosphorus insecticides by enzyme inhibition. The inhibition level can be expressed as a percentage of the hydrolysis of the substrate referred to a blank without insecticide. The percentage of hydrolysis is measured as described under Experimental.

The selection of an appropriate substrate allows the use of a flame-ionization detector instead of an electron-capture or a thermionic detector, or a flame photometric detector without loss of sensitivity. The insecticide determination is effected by an indirect method, so the insecticide residue extracts do not require elaborate clean-up to remove high levels of co-extracted material.

EXPERIMENTAL

Enzyme preparation

The enzyme suspension was obtained from fresh bovine liver tissue according to McKinley and Johal[14].

Two grams of bovine liver tissue were homogenized with 20 ml of distilled water in an ice-bath for 5 min in a CEKA homogenizer (Karl Kolb, Scientific Technical Supplies, Frankfurt am Main, G.F.R., No. 279500). After the homogenization, the suspension was centrifuged at 10,235 *g* for 20 min. The residue was discarded and the supernatant was diluted with distilled water to a final dilution of 1:200 (w/v). This enzyme preparation, when stored at 4° in a refrigerator, retained its activity for 12 days.

Enzyme substrate solution

The selected substrate was 1-naphthyl acetate, which was synthesized according to Hoogenboom[15] from 1-naphthol, acetic anhydride and sodium hydroxide (Merck, Darmstadt, G.F.R.). All chemicals used were of reagent grade. The substrate solution was prepared by dissolving 40 mg of 1-naphthyl acetate in 100 ml of ethanol (Merck) to give a concentration of 2.15×10^{-3} *M*. Then 10 μl each of tetradecane and hexadecane (Poly Science, Evanston, Ill., U.S.A.) were added to each 100 ml of substrate solution as internal standard in order for the proper range and attenuation control of the instrument to be set before the 1-naphthol and 1-naphthyl acetate peaks were eluted and recorded.

Paraoxon solutions

A set of insecticide solutions was prepared by dissolving 67.7 mg of paraoxon (Analabs, North Haven, Conn., U.S.A.) in 50 ml of distilled acetone using a 50-ml volumetric flask. A 1-ml volume of this stock solution was diluted to 1000 ml with distilled water to give a concentration of 1.35 μg/ml. Working standard solutions, containing nominally 2.7, 8.1, 13.4, 18.8, 24.2 and 32.2 ppb* of paraoxon, were prepared by diluting 0.5, 1.5, 2.5, 3.5, 4.5 and 6.0 ml of the 1.35 μg/ml solution to 250 ml with distilled water using a 250-ml volumetric flasks.

Hydrolysis reaction

The hydrolysis reaction was performed in order to determine the specific activity of the enzyme suspension. A 1-ml volume of substrate solution was incubated at 37 $\pm$ 1° in a thermostated waterbath with 0.07 ml of 1:200 (w/v) enzyme dilution, 2.0 ml of potassium phosphate buffer solution (pH 6), 2.0 ml of 0.2 *M* potassium chloride solution and 1.0 ml of distilled water. The percentage of hydrolysis was determined as described below.

Inhibition reaction

A 1-ml volume of a working standard solution of pesticide was pre-incubated in a Corning 9826 culture tube fitted with screw-cap with 0.07 ml of 1:200 (w/v) enzyme dilution, 2.0 ml of potassium phosphate buffer solution (pH 6) and 2.0 ml of 0.2 *M*

* Throughout this article, the American billion (10^9) is meant.

potassium chloride solution for 60 min at 37 ± 1°. Then, 1.0 ml of substrate solution was added and the reaction was allowed to proceed for a further 30 min at the same temperature. The reaction was stopped by extracting both the released 1-naphthol and the unhydrolyzed 1-naphthyl acetate with 2.0 ml of distilled benzene. Addition of sodium chloride crystals was necessary in order to aid the extraction. The benzene extract was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 10 drops of methanol. A 10-μl volume of this solution was injected into the gas chromatograph.

Gas chromatography

A Hewlett-Packard 5750B gas chromatograph with dual flame-ionization detectors was used. A Hewlett-Packard 3370A integrator was used to measure the peak areas.

Operating conditions

Dual stainless-steel columns (300 cm × 2 mm I.D.) packed with 1.5% OV-17 and 1.5% XE-60 on 100–120-mesh Chromosorb G were used. The oven temperature was programmed from 190° to 250° at the rate of 2°/min; the injection port was maintained at 250° and the detector block at 270°. The flow-rates were as follows: nitrogen, 20 ml/min; hydrogen, 40 ml/min; and air, 400 ml/min. All enzyme preparations were kept in an ice-bath during the assay.

The percentage of hydrolysis was calculated from the peak area data as follows:

$$\text{Hydrolysis (\%)} = \frac{\text{area of 1-naphthol peak}}{\text{area of 1-naphthol peak} + \text{area of 1-naphthyl acetate peak}} \cdot 100$$

The determination of the percentage of inhibition was accomplished with reference to a blank without pesticide as follows:

$$\text{Inhibition (\%)} = \frac{\text{\% hydrolysis of inhibition reaction}}{\text{\% hydrolysis of blank reaction}} \cdot 100$$

All of the determinations were performed in triplicate and the mean values are reported.

RESULTS AND DISCUSSION

Enzyme activity

The activity of the enzyme was determined for each new enzyme preparation. The enzyme activity is directly proportional to the amount of 1-naphthol and/or acetic acid produced by the hydrolysis reaction. The amount of 1-naphthol was calculated from the percentage of hydrolysis and the initial known amount of 1-naphthyl acetate used in the reaction.

The specific activity was expressed as moles of 1-naphthol produced by the enzyme reaction per minute per milligram of protein. The protein content of the enzyme preparation was determined spectrophotometrically according to Warburg and Christian[16], the absorbance of the enzyme suspension at 260 and 280 nm being

determined in a Beckman DU spectrophotometer fitted with a Gilford power supply and a Gilford digital visual display absorptiometer.

All of the enzyme preparation activities were slightly different from each other; the average specific activity was $27.6 \cdot 10^{-6}$ mmole of 1-naphthol per minute per milligram of protein. The constant product $27.6 \cdot 10^{-6}$ mmole of 1-naphthol per minute per milligram of protein $\times$ 0.07 ml of enzyme preparation was used to determine the correct volume of enzyme preparation to use in each experiment when the specific activity of a new enzyme preparation was too far from the average value.

The volume of enzyme preparation (0.07 ml) and the incubation time (30 min) used in all of the experiments were selected so as to ensure that 100% hydrolysis was never attained in the absence of pesticide (Fig. 1). The specific activity of the enzyme in this experiment was $27.6 \cdot 10^{-6}$ mmole of 1-naphthol per minute per milligram of protein. Under these conditions of enzyme volume and incubation time, a better resolution was obtained when the percentage of inhibition was determined.

Enzyme system

The enzyme system corresponds to a multi-enzyme system instead of a single-enzyme system. When the rate of hydrolysis was plotted against substrate concentra-

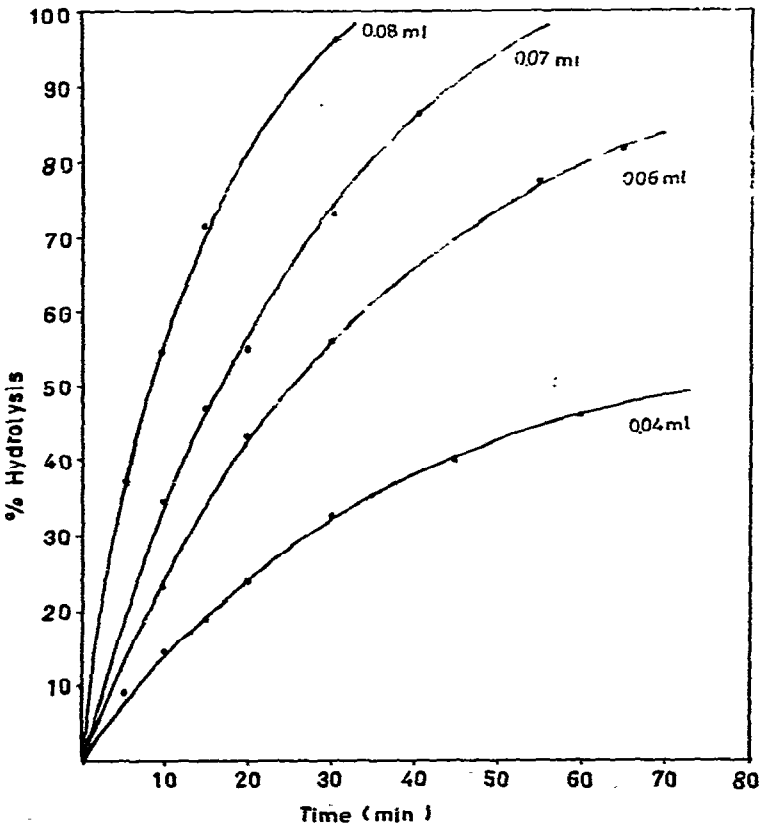

Fig. 1. Typical progress curves with different volumes of enzyme preparation.

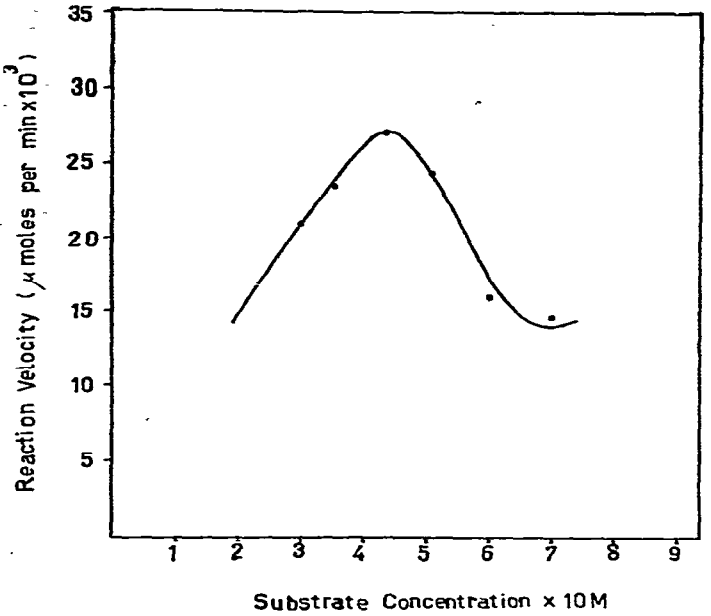

Fig. 2. Effect of substrate concentration on reaction velocity.

tion (Fig. 2), we obtained a typical graph of a multi-enzyme system[17]. This enzyme system can be fractionated by ordinary methods and the fractionation could provide more pure esterases fractions. These fractions could enhance the activity of the enzyme

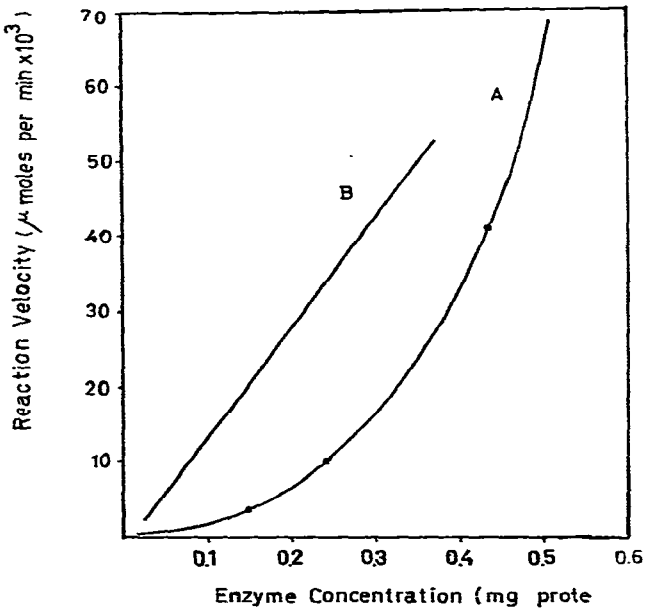

Fig. 3. Effect of activator. A, No added activator; B, activated with 0.1 M K^+.

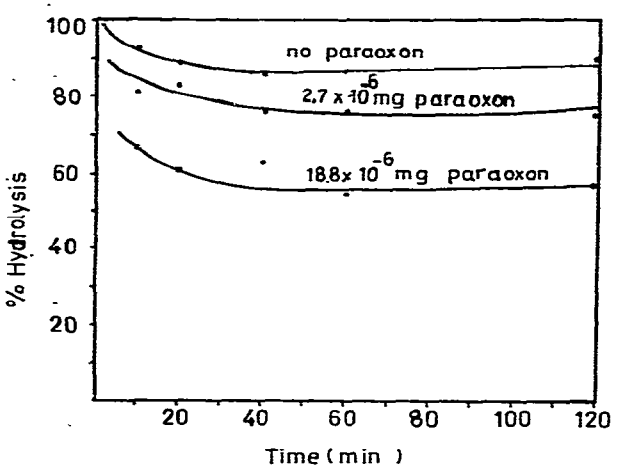

Fig. 4. Effect of time of pre-incubation of the enzyme with paraoxon on inhibition level.

reaction and the sensitivity of the inhibition test for a wide variety of organophosphorus compounds.

The studied enzyme system requires K^+ ions as an activator. The hydrolysis reaction proceeds faster in the presence of K^+ than in its absence (Fig. 3). The correct concentration of K^+ neccessary in the hydrolysis reaction was determined to be 0.1 *M* in the test-tube.

The time of pre-incubation of the enzyme with paraoxon, prior to the addition

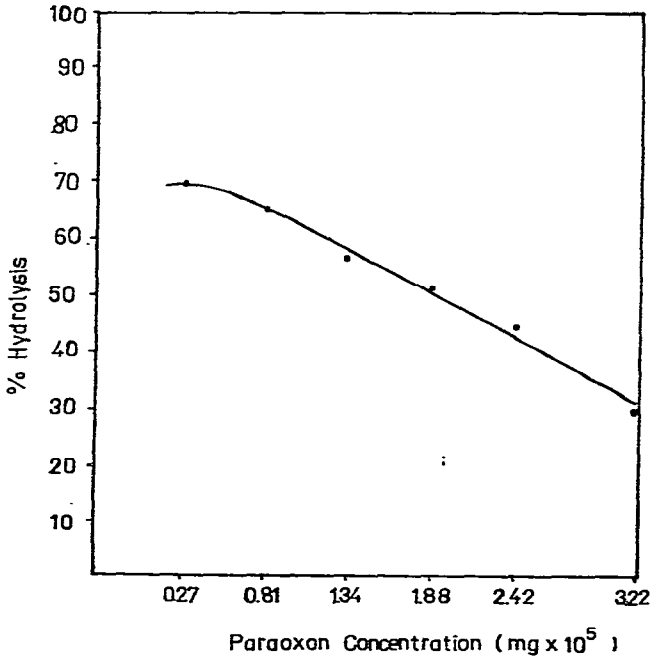

Fig. 5. Typical response curve of inhibition level with increasing amounts of paraoxon.

of substrate, was set by studying the percentage of hydrolysis of blank (Fig. 4). The percentage of hydrolysis of the blank and the inhibited reactions is independent of the pre-incubation time after 60 minutes.

The inhibition of the esterase activity by paraoxon is shown in Fig. 5. The response curve is not linear at concentrations of paraoxon below 13.4 ppb, presumably because of the multi-enzyme character of the enzyme. We have assumed that more than one enzyme with carboxylesterase activity is present in the enzyme preparation (Fig. 2). One or more of these esterases could be totally inhibited at any level of organophosphorus compounds below 13.4 ppb.

With this method it is particularly easy to perform paraoxon determinations in any kind of material and it can be applied to any other organophosphorus compound which inhibits the carboxylesterase activity.

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