Liver Esterases of Rhesus Monkey: Inhibition and Activation by Selected Pesticides

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Enzyme inhibition and activation by selected pesticides were demonstrated by a colorimetric method using *p*-nitrophenol propionate substrate and the 20,000 G fraction of the Rhesus (Rh) monkey liver extract. When 5 or 10 μl of standard pesticide solution at concentrations at or above 5×10^{-4} was used, diazinon, carbaryl, and methyl parathion strongly and selectively inhibited the liver esterases. However, methyl parathion at concentrations below 5×10^{-4} M activated the esterases as did the weak esterase inhibitor tepa at very low concentrations. The effect of certain ions on the esterase activity was also studied. NaF gave 92% inhibition of enzyme activity, while $CoCl_2$, $MnCl_2$, and $CaCl_2$ showed successively reduced inhibitory effects. $MgCl_2$ and NaCl had negligible effect on esterase activity. Starch-gel electrophoresis was also used to demonstrate the activating or inhibiting effect of the pesticides on esterases.

It is well known that organophosphorus and carbamate insecticides are strong enzyme inhibitors. However, certain chemicals that strongly inhibit esterases can also induce enzyme activity or synthesis, *in vivo*. Murphy (1966) observed that after acute poisoning of rats by organophosphorus insecticides, there was an increase in the activities of hepatic alkaline phosphatase and tyrosine-α-ketoglutarate transaminase. He also found that Delnav (*S,S'-p*-dioxane-2,3-diyl *O,O*-diethyl phosphorodithioate) had a long lasting anticholinesterase action and had produced increased and long lasting activities of hepatic alkaline phosphatase and tyrosine transaminase.

Esterase changes induced by organochlorine, organophosphorus, and carbamate pesticides were reviewed by Kay (1966). Burns and Conney (1965) reviewed the effect of certain insecticides on enzyme stimulation and inhibition in the metabolism of drugs. Morello (1965) stated that DDT probably increases the DDT-metabolizing activity of mammalian liver by inducing enzyme synthesis. Read and McKinley (1965) observed an appreciable increase in liver carboxylesterase when male rats on a vitamin A deficient diet were fed with parathion. Read *et al.* (1965) reported that liver carboxylesterase in young progeny of *p,p'*-DDT-fed rats was markedly higher than that observed in the progeny of the control rats.

Enzymes have been extracted from tissues and purified by various methods using organic solvents (Conners *et al.*, 1950; Main, 1960; Takemori *et al.*, 1967). Takemori *et al.* (1967) have demonstrated that organic solvents can stabilize the enzyme activity. Furthermore, Villeneuve *et al.* (1969) have shown that certain organic solvents applied with Ruelene (4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate) to enzyme solutions would: activate and decrease inhibition by the pesticide, deactivate and decrease inhibition, activate and increase inhibition, or deactivate and increase inhibition. It is, therefore, necessary to carefully define and control conditions when solvents are being used in the study of pesticide actions on enzyme.

This work was carried out to characterize the liver esterase activity of Rhesus (Rh) monkey towards the esters of *p*-nitrophenol (PNP) and *o*-nitrophenol (ONP) and different ions, and to demonstrate activation of esterases by certain pesticides by colorimetric method.

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EXPERIMENTAL

Enzyme Extraction. The procedure outlined in Figure 1 for extracting esterases of freshly ground Rh monkey livers was a modification of that previously reported (Mendoza *et al.*, 1969). Nicotinamide was added to the buffer to conserve the naturally occurring coenzymes during extraction. After dialysis and centrifugation, the clear 20,000 −G supernatant was used in the colorimetric studies. The protein concentration of the supernatant was 2.5 mg per ml.

Assay Procedure. The substrates used were *o*- or *p*-nitrophenyl acetate (ONPA or PNPA), propionate (ONPP or PNPP), and butyrate (ONPB or PNPB). The stock solution was 0.2 M substrate in absolute methanol, 0.05 ml of glacial

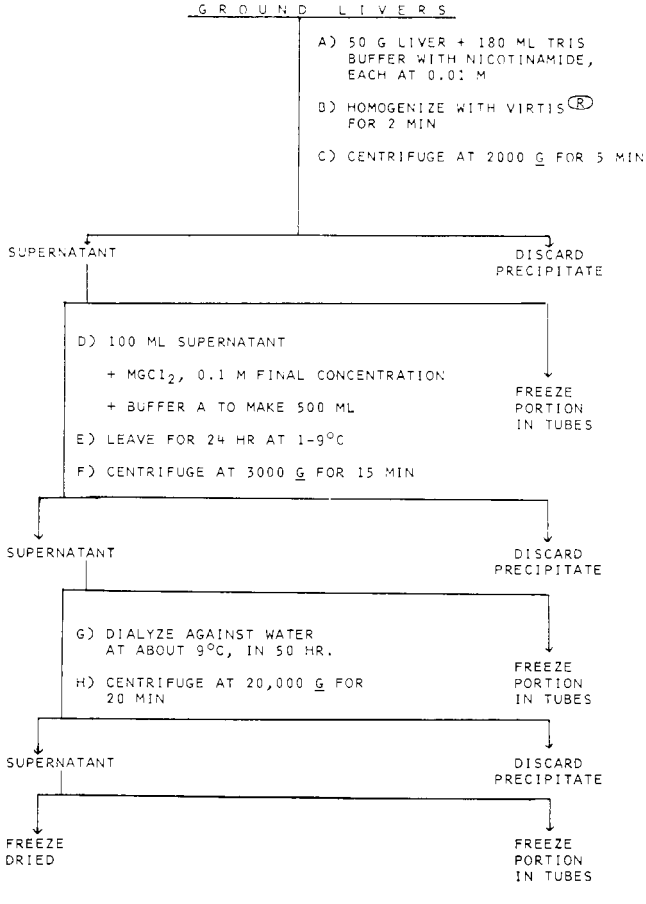


Figure 1. Outline of esterase extraction procedure

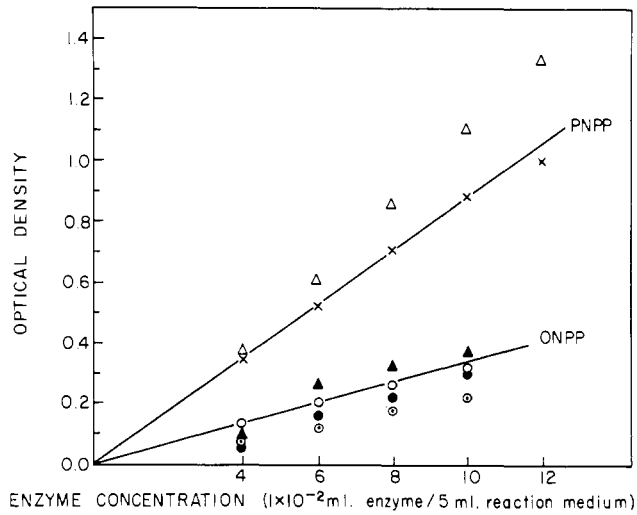


Figure 2. Comparison of hydrolyses of different esters of *o*- and *p*-nitrophenols in various enzyme concentrations read at 325 and 360 mμ

325 mμ: × = PNPP; ● = PNPA; △ = PNPB; 360 mμ: ○ = ONPP; ⊙ = ONPA; ▲ = ONPB

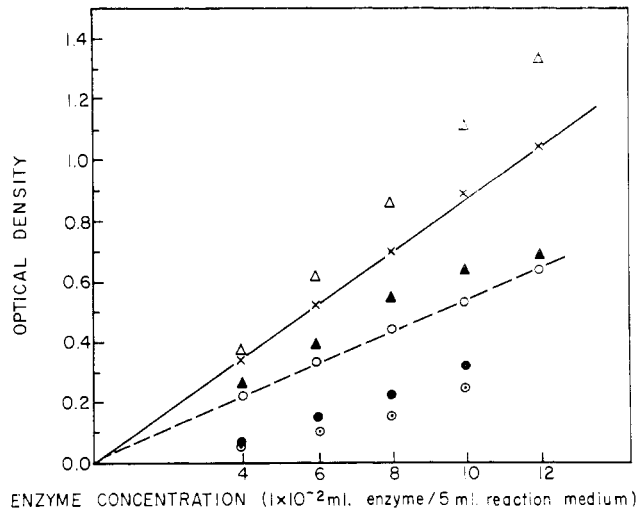


Figure 3. Comparison of hydrolyses of *p*-nitrophenol esters read at 325 and 347 mμ

325 mμ: × = PNPP ● = PNPA; △ = PNPB; 347 mμ: ○ = PNPP ⊙ = PNPA, ▲ = PNPB

acetic acid per 10 ml of stock solution was added to reduce the rate of substrate decomposition. Before use in assay, 0.2 ml of the stock solution was diluted with 1 ml of absolute methanol and was finally made to 0.4 mM by diluting the solution with 0.01 M tris-maleate buffer at pH 6.3.

Before use, the 20,000 −G supernatant (enzyme solution) was diluted 25 times with 0.01 M tris-maleate buffer at pH 6.3.

The rate of hydrolysis of different substrates was determined spectrophotometrically. Three ml of the diluted enzyme solution was mixed with 2 ml of 0.4 mM substrate and was incubated at 37° C for 15 min. The reaction was then stopped by adding 0.05 ml of 6 N HCl; the color due to hydrolysis of PNP or ONP from the carboxylic esters was measured by a Beckman DU spectrophotometer.

The effect of certain ions on enzyme activity was studied by adding 10 μl of 1.0 M salt solution to 3 ml of diluted enzyme. The solution was incubated at 37° C for 15 min before adding 2 ml of 0.4 mM PNPP solution. After 15 min, the reaction was stopped with 0.05 ml of 6 N HCl and the amount of PNP hydrolyzed was measured.

To study the effect of pesticides, different concentrations of carbaryl (1-naphthyl methylcarbamate, 99.7% purity); diazinon [*O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothioate, 95.8%]; methyl parathion (*O,O*-dimethyl-*O*-*p*-nitrophenyl phosphorothioate, 99.4%]; and tepa (tris (1-aziridinyl) phosphine, 100%), each in 5 or 10 μl of absolute methanol, were added to 3 ml of enzyme solution. The resulting solution was incubated at 37° C for 30 min before adding 2 ml of 0.4 mM PNPP solution. After 15 min, 0.05 ml of 6 N HCl was added to stop the reaction and the amount of PNP hydrolyzed was measured.

The effect of pesticides on liver esterases was also studied by starch-gel electrophoretic procedure reported by Mendoza and Hatina (1970). Before carrying out the electrophoretic procedure, the 2000 and 20,000 −G esterase fractions were incubated at 37° C for 30 min with each of the pesticides. One part of the pesticide solution at 5×10^{-2} or 5×10^{-6} M was added to nine parts of 2000 G solution or 20,000 −G freeze-dried supernatant that was redissolved in distilled water at 15 mg to 1 ml of water. The treated and the control liver extracts were applied with uniform sized filter paper into the gel slits 5 cm from the cathodic edge of the gel. The gel used was 8 × 108 × 186 mm. Electrophoresis was carried out 160 v for 15 min before the paper strips were removed and at 300 v until the brown borate front (Mendoza and Hatina, 1970) traveled 8 cm from the slits. The developed gel was stained with α-naphthyl acetate.

RESULTS AND DISCUSSION

Comparison Among ONP and PNP Esters. Figure 2 shows the comparison among ONP and PNP esters. The esters with the nitro group at the *para* position in the phenyl ring were more readily hydrolyzed than those with the nitro group at the *ortho* position, indicating that the former were perferable substrates for the esterase. PNPP and PNPB were hydrolyzed much faster than ONPA, PNPA, ONPP, and ONPB. ONPP gave a linear relationship with the enzyme concentrations used, although it was hydrolyzed more slowly than the ONPB.

Comparison Among PNP Esters. Figure 3 shows that PNPB and PNPP were hydrolyzed readily by the monkey liver esterase. Under the conditions used, the amount of PNPP hydrolyzed determined at 325 mμ maximum or at 347 mμ isosbestic point increased linearly as the enzyme concentrations increased. Likewise, the amount of PNPB hydrolyzed determined at 325 mμ increased linearly as the enzyme concentrations increased. However, the same PNPB reaction solutions measured at 347 mμ showed a nonlinear increase of PNPB hydrolyzed as the enzyme concentrations increased. PNPA was hydrolyzed at the slowest rate amongst substrates tested, although the amount hydrolyzed increased linearly as the enzyme concentrations increased. Because of the unexplained changes in the absorption of PNP when PNPB was used as a substrate, and because PNPA was more slowly hydrolyzed, PNPP was used in the subsequent experiments.

Effects of Ions on Enzyme Activity. It is known that certain ions may act as cofactors or can decrease the enzyme activity. Figure 4 shows the effect of certain ions on the enzyme activity; in each 5 ml reaction medium, the salt concentration was 2×10^{-3} M. NaF gave the strongest

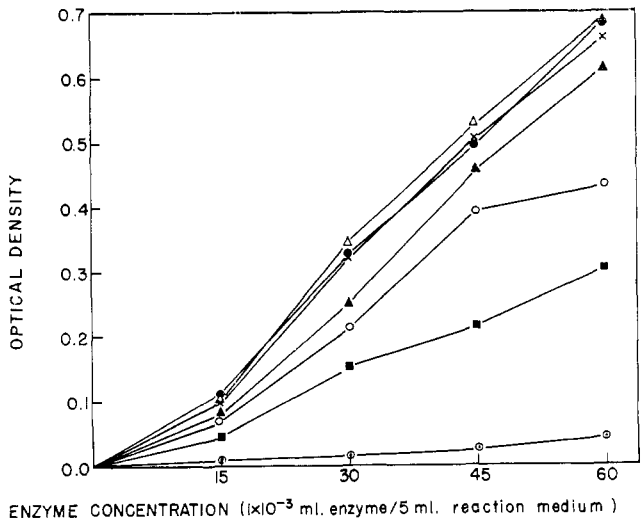


Figure 4. Effect of different ions on the hydrolysis of PNPP in various enzyme concentrations: the control plotted is lower than NaCl and $MgCl_2$ because it is the mean of all controls for each chemical used and includes variation between controls

× = blank, ● = NaCl, ○ = $MnCl_2$, △ = $MgCl_2$, ▲ = $CaCl_2$, ⊙ = NaF, ■ = $CoCl_2$

inhibition of esterases, an average decrease in enzyme activity of 92% when compared with the control. It was followed by $CoCl_2$ (38–50% inhibition), $MnCl_2$ (33%), $CaCl_2$ (17%), $MgCl_2$ (5%), and NaCl (0.2%). At the highest concentration of enzyme (0.06 ml/5 ml reaction medium), NaCl and $MgCl_2$ produced negligible effect. NaCl at 0.02 M was used in the medium for enzyme inhibition by pesticides, but showed no advantage over the use of buffer alone.

Effect of Pesticides on Enzyme at Various Concentrations. Figure 5 shows the effect of increasing amount of enzyme on the inhibition by pesticides at a constant concentration,

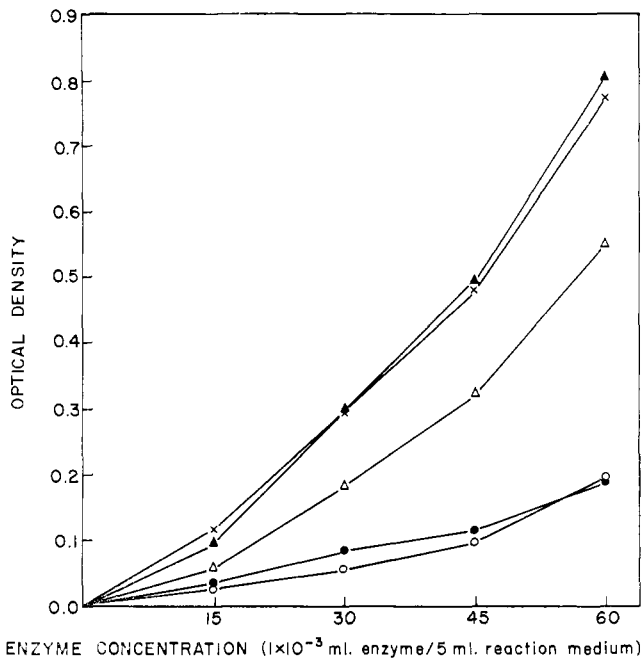
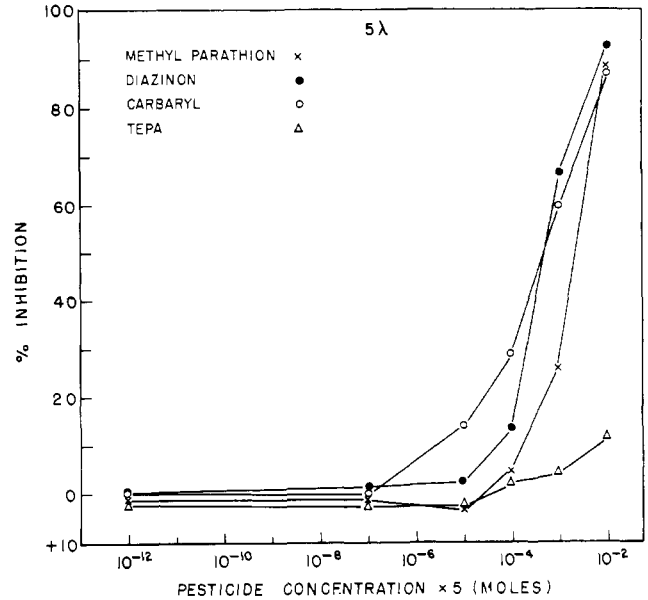


Figure 5. Effect of different pesticides on the hydrolysis of PNPP in various enzyme concentrations

× = control, ● = carbaryl, ○ = diazinon, △ = methyl parathion, ▲ = tepa



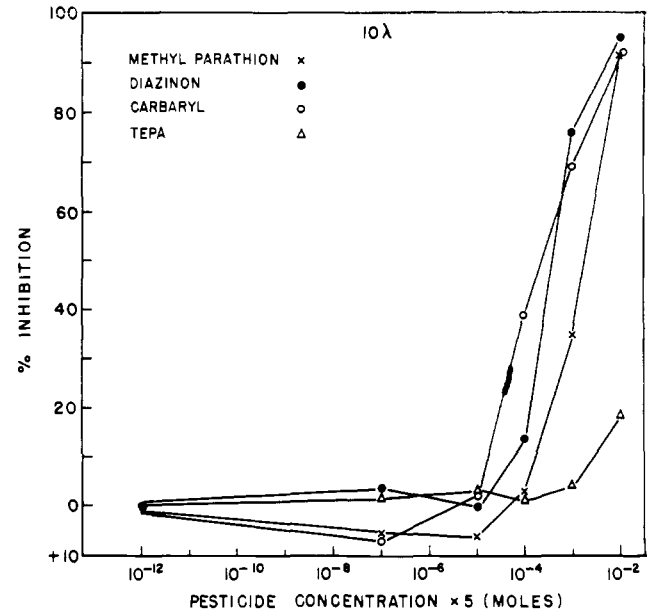


Figure 6. Effect of different concentrations of pesticides on enzyme at constant concentration, temperature, and pH

Below zero inhibition indicated enzyme activation. Pesticides applied in (A) 5 μl and (B) 10 μl of methanol

Diazinon and carbaryl gave the greatest enzyme inhibition, followed by methyl parathion and then by tepa. At high enzyme concentrations used, tepa had no effect on the esterase activity.

Effects of Different Levels of Pesticides on the Enzyme Activity. Figure 6 shows that strong inhibition Rh monkey liver esterases was obtained with 5×10^{-2} M of diazinon, methyl parathion, or carbaryl; the pesticides were enumerated at the decreasing order of inhibition. Tepa gave the least enzyme inhibition. As concentrations of the standard, diazinon and carbaryl decreased from 5×10^{-2} to 5×10^{-4} M, the decrease in percentage inhibition was smaller than the decrease observed when the concentration of methyl parathion was changed from 5×10^{-2} to 5×10^{-4} M. At 5×10^{-3} M, diazinon and carbaryl still showed some inhibition of esterases, whereas methyl parathion showed marked activation. The difference in the degree of enzyme

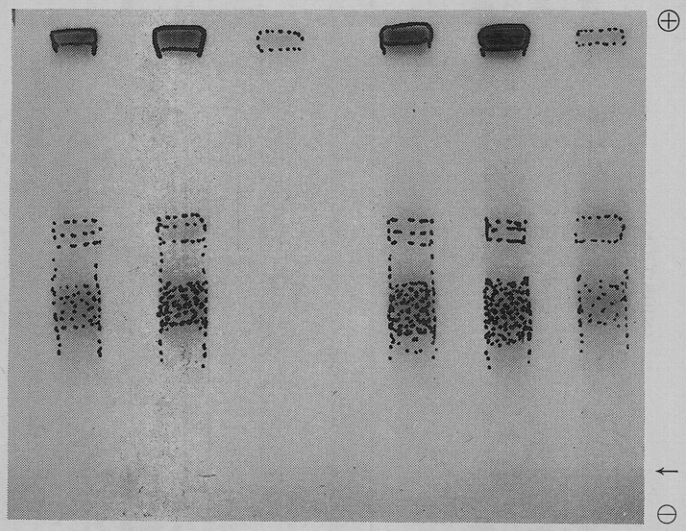


Figure 7. Typical electrophoregram of the 20,000 G Rh monkey liver esterase showing enzyme activation due to certain pesticides

(1) control, (2) 5×10^{-7}-M, and (3) 5×10^{-3}-M methyl parathion treatments, (4) control, (5) 5×10^{-7}-M, and (6) 5×10^{-3}-M carbaryl treatments. The arrow points at the application slit. The tris-HCl buffer used for starch-gel preparation was at 0.01M and pH 7.5. α-Naphthyl acetate was used as a substrate. (Note the intensity of the fastest band of the enzyme treated with pesticides, each at 5×10^{-7} M, located at the 2nd and 5th slots)

inhibition at certain levels of diazinon, carbaryl, methyl parathion, and tepa indicated specificity in enzyme-pesticide interaction. This was further substantiated by carbaryl, which was a stronger inhibitor than any of the pesticides when used at 5×10^{-5} and 5×10^{-4} M. Methyl parathion activated the esterase when it was applied with 5 or 10 μl of methanol. With tepa, consistent activation was observed only when this pesticide was in 5 μl of methanol, indicating that the amount of methanol might have influenced the enzyme activation. This observation concurred with the previous reports (Villeneuve *et al.*, 1969; Mendoza and Hatina, 1970) that solvents could affect the activity of the enzyme. The relation of activation to the pesticide concentration was not linear, but carefully controlled experiments confirmed that activation was consistent only when methyl parathion and tepa were present at low concentrations. Although some activation of esterases by diazinon and carbaryl was observed, the effect was sporadic and did not exhibit a consistent trend.

Starch-Gel Electrophoresis. Starch-gel electrophoresis with α-naphthylacetate as a substrate was also used to study the effect of pesticides on liver esterases. The 2000 −G esterase fraction was incubated at 37° C for 30 min with each of the pesticides. At 5×10^{-7} M, methyl parathion, diazinon, and carbaryl did not show any marked effect on enzymes. At 5×10^{-3} M, carbaryl and diazinon selectively inhibited the fast-moving band while methyl parathion and tepa had no effect on the enzymes.

However, when the 20,000 −G esterase fraction was incubated at 37° C with methyl parathion, carbaryl, and diazinon, each at 5×10^{-7} M, slight activation of the esterase fast-moving band towards α-naphthyl acetate was observed, Figure 7. Tepa at 5×10^{-7} M produced negligible effect. The esterase was inhibited by the following pesticides given at the decreasing order of inhibitory effect: methyl parathion, diazinon, carbaryl, and tepa, each at 5×10^{-3} M.

CONCLUSIONS

The results demonstrated that certain pesticides that are strong inhibitors at high concentrations can be activators at a very low level. The decrease in the enzyme inhibition by methyl parathion as its concentration decreased may be due to the activation effect at lower concentrations. The esterase inhibition or activation by pesticides determined by the colorimetric method using PNPP can also be determined by starch-gel electrophoresis. Further studies of other pesticides at lower concentrations will be of value in characterizing or grouping the pesticide residues in conjunction with the existing methods.

Whether or not there is an increase in the degradation rate of pesticides when the enzymes are activated has yet to be investigated. Also, the biological significance of the activating effect at the low pesticide dosage is not known.

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