Separation and Purification of DDT-Degrading Enzymes from the Human Body Louse

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Purified DDT-degrading fractions have been separated from aqueous extracts of human body louse by tertiary amyl alcohol. Evidence that this has occurred is the identification of reaction products produced by each fraction. Purification of the fractions was obtained by calcium phosphate gel adsorption and subsequent elution, acetone fractionation, and chromatography on hydroxylapatite. The crude fractions are heat stable and lose this property upon purification. Based on the various metabolites produced, several enzyme types are considered to occur naturally and are not the result of degradation of a single original native enzyme.

In recent years, several papers have appeared on methods of purification and measurement of DDT dehydrochlorinase (*3*, *5*). This enzyme, found in DDT-resistant houseflies, converts 1,1,1 - trichloro - 2,2 - bis(*p* - chlorophenyl)ethane (DDT) to its nontoxic ethylene derivative 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene (DDE). The detoxification enzyme apparently plays a vital role in the resistance of houseflies to DDT. Tombes and Forgash (*11*) described a similar enzyme present in the various life stages of the Mexican bean beetle. Also, Agosin *et al.* (*1*) reported that an enzyme system exists in the tissue of German and American roaches capable of converting DDT to 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethanol.

Perry and Buckner (*8*) found an enzyme system in the human body louse which degrades DDT in a manner different from housefly DDT-dehydrochlorinase. The present paper describes methods and procedures used in the purification and separation of enzymes in the human body louse which degrade DDT.

Properties of enzymes as well as methods of assay are presented. Characterization of DDT degradation products is described in another paper (*9*).

Materials

A DDT-resistant strain and a susceptible strain of Korean body lice were generously provided by the Entomology Research Station, U.S.D.A., Orlando, Fla. Hydroxylapatite was prepared according to the method of Tiselius, Hjerten, and Levin (*10*). Crystalline trypsin and bovine serum albumin were purchased from the Nutritional Biochemical Corp. Glutathione was purchased from Sigma Chemical Co. C^{14}-DDT, 160 μc. per mole, was prepared in this laboratory (*6*, *7*).

Table I. Summary of Purification Procedure for Enzyme Fraction *A*

Fraction	Total Volume, Ml.	Units[a] per Ml.	Total Units	Protein, Mg. per Ml.	Specific Activity	Purification	Yield, %
Homogenate	90	70	6300	13.13	5.3	...	100
t-Amyl alcohol	87	56	4872	2.37	24.00	4.5	80
Calcium phosphate gel	84	60	5040	0.44	136.0	25.4	85
Heat and acetone	20	54	1080	0.34	157.0	29.4	77
Hydroxylapatite chromatography	17	28	476	0.04	640	120	40

[a] Based on total micrograms of neutral and acidic metabolites produced by enzyme fraction *A*.

Table II. Summary of Purification Procedure for Enzyme Fraction *B*

Fraction	Total Volume, Ml.	Units[a] per Ml.	Total Units	Protein, Mg. per Ml.	Specific Activity	Purification	Yield, %
Homogenate	90	71	6390	13.13	5.40	..	100
t-Amyl alcohol	87	71	6177	3.36	21.20	3.9	100
Calcium phosphate gel	84	62	5208	1.27	48.80	9.0	88
Acetone and heat	20	54	1080	1.05	51.50	9.6	76
Hydroxylapatite chromatography	17	41	697	0.17	234.0	43.5	58

[a] Based on total micrograms of neutral and acidic metabolites produced by enzyme fraction *B*.

Methods

Enzyme Assays. Enzyme reactions were carried out in a Dubnoff shaker at 37° C. under atmosphere of nitrogen. The enzymatic reaction requires anaerobic conditions. Approximately 150 μg. of C^{14}-DDT in 100 μl. of acetone were added to 2 ml. of enzyme preparation. The volume of the mixture was brought to 2.5 ml. by the addition of 0.5 ml. of tris-HCl buffer pH 8.4. The reaction mixture was gassed for 15 minutes with nitrogen and simultaneously equilibrated to the bath temperature. At the end of this time, 4 mg. of glutathione in 0.5 ml. of 0.02*M* sodium bicarbonate was added to the mixture. The routine reaction time was 2 hours.

For analysis, the incubation mixture was transferred quantitatively from the reaction beakers to glass-stoppered flasks. This was followed by the addition of an equal volume of hexane, and the flasks were shaken vigorously for at least 20 minutes on a wrist-action shaker to complete extraction. The mixture was transferred to centrifuge tubes and centrifuged 4000 × G for 10 minutes to effect complete separation of the two layers. Unreacted DDT passed into the hexane layer, and the polar metabolites remained in the water layer. The two layers were separated by siphoning, and quantitative determinations of C^{14} were made in a liquid scintillation counter (*9*).

Protein Determination. Protein content of crude preparations was estimated by the micro-Kjeldahl method and that of the purified preparations by

the method of Lowry *et al.* (*4*). Crystalline bovine serum albumin served as a standard. One unit of enzyme is defined as the quantity of enzyme required for the degradation of 1 μg. of DDT under the conditions of assay. Specific activity is expressed as number of enzyme units per mg. of protein.

Purification of the DDT-ases

Preparation of Homogenate. Eight per cent (w./v.) tissue homogenates were prepared by triturating the lice in a mortar with a 1:1 mixture of 0.1*M* NaCl and 0.1*M* $NaHCO_3$ and homogenizing in a Potter-Elvehjem glass homogenizer. This was followed by blending in a Servall Omni-mixer operating at a speed of 14,000 r.p.m. for 2 minutes. Gross particles were then removed by filtering through surgical cotton gauze. The homogenate was centrifuged at 20,000 × G for 20 minutes, and the supernatant liquid was carefully decanted so as not to disturb the sedimented particles. The DDT-ase activity remained in the supernatant fraction (crude extract). When such enzyme preparations are kept below 2° C., activity can be maintained for months.

Tertiary Amyl Alcohol Fractionation. Ninety milliliters of centrifuged homogenate was chilled in an ice bath, and 20 ml. of *t*-amyl alcohol at −2° C. was added dropwise with stirring. The solution was stirred well and the temperature was not allowed to rise above 2° C. Stirring was continued for another 30 minutes and the mixture centrifuged at 24,000 × G for 20 minutes. The precipitate was dissolved in 0.05*M* phosphate buffer, pH 8.0, and designated as enzyme fraction *A*. The supernatant fraction was designated as enzyme fraction *B*. Both fractions possessed approximately the same enzyme activity as the original centrifuged homogenate. This is shown in Tables I and II. Thus. treatment with this solvent resulted in an unmasking of activity which was not evident in the crude extracts. Both enzyme fractions *A* and *B* were dialyzed overnight at 0° C. against 0.02*M* phosphate.

Trypsin Digestion. The fractions were removed from the dialysis sacs, and 100 μg. of trypsin was added to each ml. of enzyme fraction. The mixtures were stirred continuously for 3 hours at room temperature. The trypsin yielded solubilized preparations making it possible to use organic solvents for further purification. The fractions were then subjected to further dialysis to remove the last traces of tertiary amyl alcohol.

Calcium Phosphate Gel Adsorption and Elution. The gel was prepared by the method of Keilin and Hartree (*2*). Calcium phosphate gel was added to both enzyme fraction *A* and enzyme fraction *B* using 10 mg. of gel per ml. of enzyme fraction. The mixtures were stirred for 30 minutes at 0° C. and then centrifuged. The supernatant liquids were discarded. The gel carrying the enzyme activities was suspended in 0.02*M* phosphate buffer and recentrifuged. The supernatant liquid was discarded. The enzymes were removed from the gel by homogenizing in a saturated solution of tetrasodium pyrophosphate. Salts were then removed from the solutions by dialysis at 2° C. for 8 hours against a 0.02*M* phosphate buffer pH 8.2 No activity was lost upon dialysis.

Heat Treatment at pH 8.2. Each fraction was placed in a beaker in a water bath. Under constant vigorous stirring, the temperature of the fractions was brought to 65° C., held within 60° to 65° C. for 15 minutes, and then cooled rapidly in an acetone–dry ice bath at −5° C. This was followed by centrifugation at 12,800 × G for 10 minutes and the clear supernatant, liquid was saved. Heating to 65° C. removed inactive protein.

Acetone Fractionation. The temperature of enzyme fraction *A* and enzyme fraction *B* was lowered to 0° C. For each 10 ml. of enzyme fraction *A*, 4 ml. of acetone, chilled to −10° C., was added with continuous stirring. Likewise, for each 10 ml. of enzyme fraction *B*, 5 ml. of acetone was added under the same conditions. Stirring was continued for 15 minutes after the addition of acetone and was followed by centrifugation for 20 minutes at 20,000 × G. The precipitate was discarded since it had no enzyme activity.

Chromatography on Hydroxylapatite. Further purification was achieved by chromatography on hydroxylapatite. The hydroxylapatite was packed into a column (1 × 10 cm.) and equilibrated with 0.02*M* phosphate buffer pH 8.2. The enzyme fractions were placed on the column and washed with the same buffer. Through pressure maintained by compressed nitrogen, a flow rate of 4 to 6 ml. per hour was used. Elution of enzyme fraction *A* was effected by a saturated solution of tetrasodium pyrophosphate. Enzyme fraction *B* was eluted with 0.2*M* phosphate buffer. The column yielded fractions of high specific activity. The purification achieved and the recovery of enzyme fraction *A* and enzyme fraction *B* are given in Tables I and II. Other methods of purification such as fractionation on DEAE cellulose and ammonium sulfate were not effective.

Results

Identification of Reaction Products. Enzyme fraction *A* gave a predominantly neutral metabolite which had an R_f value identical to DDE. The ultraviolet absorption spectrum and infrared spectrum of the metabolite were similar to DDE. Enzyme fraction *B* gave a predominantly neutral metabolite which had an R_f value identical to 4,4′-dichlorobenzophenone. The ultraviolet absorption spectrum and the infrared spectrum were the same as the aromatic ketone. Both enzyme fractions gave an acidic metabolite tentatively identified as bis-(*p*-chlorophenyl)acetic acid (DDA) (*9*).

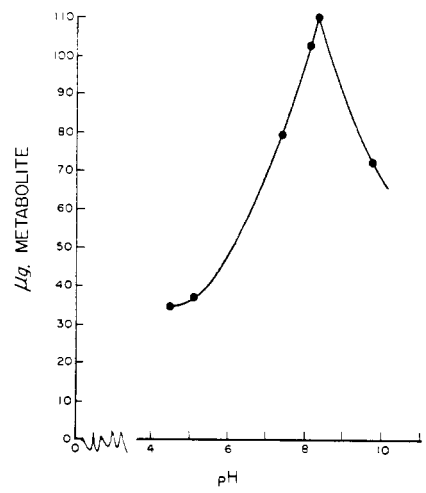

Figure 1. The effect of pH on activity of enzyme fraction *A*

Reaction mixture: 2 ml. enzyme, 160 μg. C^{14}-DDT; 3×10^{-3}M GSH under N_2 atmosphere. Buffers used: acetate from pH 4.0 to 5.5, phosphate from pH 6.0 to 8.0, tris-HCl from 8.0 to 9.0, and carbonate-bicarbonate from 9.0 to 10.5

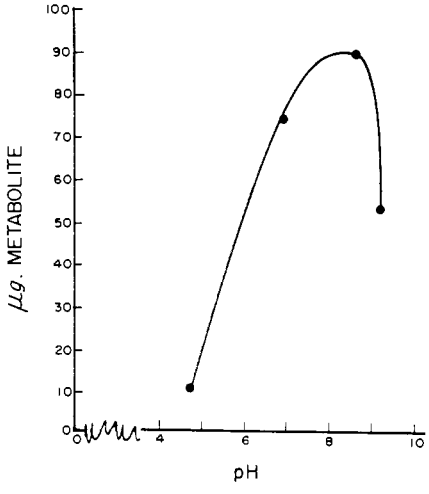

Figure 2. The effect of pH on activity of enzyme fraction *B*

Reaction mixture: 2 ml. enzyme, 160 μg. C^{14}-DDT; 3×10^{-3}M GSH under N_2 atmosphere. Buffers used: acetate from pH 4.0 to 5.5, phosphate from pH 6.0 to 8.0, tris-HCl from 8.0 to 9.0 and carbonate-bicarbonate from 9.0 to 10.5

Properties of Enzyme Fractions

Stability of Enzyme Fractions. Crude preparations and products obtained after all purification steps up to

and including calcium phosphate gel treatment could be preserved at 0° in 0.02*M* phosphate buffer pH 8.2 with no loss in activity. Variation in the pH over the range 8.0 to 8.6 had little effect on enzyme stability. Outside this range the rate of inactivation increased. Preparations after the acetone step in purification were less stable than those of the crude extract. For example, a sample lost 20% of its initial activity after standing several days at 2° C. The crude extract and preparations up to and including calcium phosphate gel are relatively heat stable for there is only slight destruction of enzyme activity after heating 15 minutes at 65° C. Heat treatment enabled the use of larger amounts of acetone without objectionable loss of activity in the acetone purification step.

Effect of Metals and Sulfhydryl Compounds. Attempts were made to stimulate enzyme activity by the addition of metal ions. At $10^{-3}M$, Mg^{+2}, Ni^{+2}, Pb^{+2}, Mn^{+2}, Fe^{+2}, and Co^{+2} were without effect. The enzyme fractions were inhibited by $10^{-2}M$ *p*-hydroxymercuribenzoate and iodoacetate. This evidence clearly indicates that both enzyme fractions are sulfhydryl-dependent enzymes. In the presence of either inhibitor, approximately 20% of the enzyme activity remained. Also, under anaerobic conditions and in the absence of glutathione, 13% of the enzyme activity was present. In keeping with these observations, glutathione, cysteine, and thioglycollic acid stimulated the enzyme activity.

Discussion

In the housefly, the only product obtained from the attack of DDT-dehydrochlorinase on DDT is DDE. The human body louse has enzymes which produce a number of DDT-degradation products. The present results indicate that these native enzymes are a mixture of several types. The partial separation of these types was effected by the use of *t*-amyl alcohol. Each type apparently produces a different metabolite. Enzyme fraction *A* gave a 6:1 ratio of neutral to acidic metabolite. The neutral metabolite was predominantly DDE. Similarly, enzyme fraction *B* gave a 1:1 ratio of neutral to acidic metabolite. The neutral metabolite in this case was predominantly 4,4′-dichlorobenzophenone. The acidic metabolite produced by both enzyme fractions appears to be DDA, since the metabolite has the same R_f value and similar infrared spectrum as DDA.

Additional evidence on identification of this metabolite awaits further purification. The results of the identification of metabolites have been significant and seem to indicate that the multiplicity of enzyme types is the consequence of the actual occurrence of various DDT-degrading enzymes in the human body louse. Each enzyme fraction was solubilized by the addition of trypsin. This treatment made it possible to use organic solvents at higher concentration than usual thus effecting a greater degree of purification. No appreciable purification resulted from the use of acetone before trypsin treatment in spite of the great stability of the enzymes in the presence of this solvent. Like most enzymes, DDT-degrading enzymes have a narrow pH range through which they exert their maximum effect. This is shown in Figures 1 and 2. The crude extract is heat stable, although upon purification with acetone it loses this property. The cause for the change in heat stability is not known. The influence of enzyme concentration and substrate concentration was like that of most enzymes, the rate of activity being proportional to enzyme concentration and to the substrate concentration up to a limiting value (*9*). Study of the enzyme substrate relationships in greater detail must await further separation of enzyme types (*9*).

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INSECTICIDE DECOMPOSITION

Nuclear Magnetic Resonance in the Examination of the Thermal Decomposition of *O,O*-Dimethyl *O*-[4-(Methylthio)-3-tolyl] Phosphorothioate

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During investigations on the metabolic fate of *O,O*-dimethyl *O*-[4-(methylthio)-3-tolyl] phosphorothioate (Baytex) in plants and its breakdown on plant surfaces upon exposure to sunlight and air, it became necessary to identify some of the products which occur under these conditions. Because heat applied to some phosphorothioic acid esters often gives the same products as does irradiation, the effects of heat on Baytex were examined.

Finegold (*2*) showed that the NMR (nuclear magnetic resonance) chemical shift values of P^{31} nuclei are unique for each type of phosphorus compound and vary markedly, depending upon the nature of the group on the phosphorus atom. Therefore, P^{31} NMR spectra can be used to detect and identify the presence of various types of phosphorus compounds in a mixture, provided their concentration exceeds 3 to 10%—the limit of the sensitivity of the instrument. However, P^{31} chemical shift values will differ only when there is a change in the molecular environment just adjacent to the phosphorus atom—e.g., the isomerization of a phosphorothionate to a phosphorothiolate, and no significant variation in the P^{31} signal is found when the change is two bonds removed from the phosphorus atom. Changes in substituents farther removed from phosphorus, however, can be detected by proton (H^1) magnetic resonance spectra, and both P^{31} and H^1 spectra of Baytex were examined after various intervals of heating at different temperatures.