DDT Dehydrochlorinase

I. ISOLATION, CHEMICAL PROPERTIES, AND SPECTROPHOTOMETRIC ASSAY*

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The dehydrochlorination of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane to its nontoxic, ethylenic analogue, DDE,† by extracts of DDT-resistant houseflies (Musca domestica) was first demonstrated by Sternburg et al. (1). It was shown that this system was GSH-dependent and that no DDT dehydrochlorinase could be detected in DDT susceptible flies by the means then available. The titer of DDT dehydrochlorinase in various strains of flies was directly proportional to the degree of resistance to DDT (2), and it was concluded that the presence of this detoxicating system was a factor of major importance in the survival of populations of houseflies exposed to DDT. The reaction is as follows:

\[
(p\text{-C}_6\text{H}_4\text{Cl}_3)\text{C} - \text{CH}_2\text{Cl}_2 \xrightarrow{\text{GSH}} (p\text{-C}_6\text{H}_4\text{Cl}_3)\text{C} - \text{CCl}_2 + \text{H}^+ + \text{Cl}^-
\]

The initial studies on the nature of DDT dehydrochlorinase relied on assays in which the substrate, DDT, was incorporated in the system in a particulate form, either in an ethanol-water emulsion, or on DDT-coated glass beads (1–3). This paper describes a direct spectrophotometric assay suitable for kinetic measurements using egg yolk lipoprotein as a solubilizing agent. A method for the purification of the enzyme is also presented along with some properties of the enzyme. Substrate and cofactor specificity will be described in the following paper (4).

**EXPERIMENTAL**

**Methods**—At specific activities > 0.05 protein was determined by biuret as modified by Robinson and Hogden (5). At higher purities the spectrophotometric method is suitable with \(E_{278}^{10} = 9.8\).

**Spectrophotometric Assay of DDT Dehydrochlorinase with Lipoprotein-solubilized DDT**—At 25°C the solubility of DDT in water is of the order of \(10^{-4}\) m mole per ml. (6). Although the product of DDT dehydrochlorinase action, DDE, exhibits solubility of a similar magnitude, its strong absorbance in the 250 to 280 nm region of the spectrum (2) suggested that a direct spectrophotometric assay could be devised, providing solubilization of the substrate and product could be achieved. The need for a rapid assay suitable for kinetic studies was made even more urgent when it was observed that preparations of specific activity >0.05 no longer gave a linear response of activity versus protein concentration when measured by the glass bead method, presumably due to sorption of the enzyme on the beads. The use of a relatively mild solubilizing agent such as lipoprotein was indicated when preliminary studies showed that the surface active agents commonly used with water-insoluble substrates rapidly inactivated the enzyme. These included digitonin, lecithin, ionic and nonionic synthetic detergents, and mixed solvent systems of water and ethanol, butanol, dimethylformamide, or ether.

A modification of the ultracentrifugal flotation technique of Dalalla and Gofman (7) was developed by Crespi (8) which provided unlimited quantities of the 8% fraction of egg yolk lipovitellin. This material held about 12 m moles of DDT per ml. The turbidity of this material precluded its use in the spectrophotometer, but it may be used for solubilization of materials where an optically clear preparation is not needed. Clarification can be effected by extraction with ether in the cold. The limitations of the spectrophotometric assay are the converse of the bead assay, enzyme preparations of specific activity <0.03 can not be measured directly in the spectrophotometer due to the opacity of such crude preparations in the 260 to 280 nm region.

**Preparation of Lipoprotein**—The yolks of 5 fresh eggs are rinsed free from the white and suspended in 4 per cent Na2 citrate at 20°C. Each gm. of yolk is suspended in 5 gm. of citrate solution (containing 500 m g. of neutralized Versene (ethylene-diaminetetraacetate) per ml.). Sufficient material is available for the full capacity of the No. 30 rotor of the Spinco model L centrifuge. The lipoprotein is centrifuged for 19 hours at 80,000 \(\times g\) at 2°C and the clear lipovitellin gel at the meniscus of the tubes is removed and suspended in 75 ml. of 4 per cent citrate. The preparation is brought to 2°C and extracted briefly and gently with 50 ml. of precooled ethyl ether. Four slow inversions of the separatory funnel are sufficient to remove the turbidity from the aqueous phase. The lipovitellin is removed after 24 hours and freed from all but a trace of ether in a vacuum. The straw-colored stock solution of lipoprotein can be stored at 2°C for 4 to 5 months.

**Preparation of Lipoprotein-DDT**—A 25-ml Erlenmeyer flask is prepared containing 1 gm. of pavement marking beads coated with 20 mg. of DDT in the usual manner (2). A 10 ml aliquot of the stock lipoprotein solution is freed from the last traces of...
TABLE I

Components of lipoprotein-DDT assay system for DDT dehydrochlorinase

The system contained DDT or DDE, 0.24 μmole in 4.6 mg of lipoprotein; GSH, 13 μmoles; DDT dehydrochlorinase, 0.18 unit; phosphate, 275 μmoles, pH 7.4. Incubated at 25°, 8.5 minutes.

<table>
<thead>
<tr>
<th>System</th>
<th>ΔE₅₆₀</th>
<th>ΔEm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete (DDT)</td>
<td>0.158</td>
<td>0.002</td>
</tr>
<tr>
<td>No GSH</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>No DDT</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>No lipoprotein</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>No DDT dehydrochlorinase</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Boiled DDT dehydrochlorinase</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Complete (DDE)</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Initial rate of DDT dehydrochlorinase reaction with lipoprotein-DDT. The system contains DDT dehydrochlorinase, 0.3 unit; GSH, 10 μmoles; DDT, 0.3 μmole in 4 mg lipoprotein; phosphate, 275 μmoles, pH 7.4; volume, 3.5 ml. Measured at 25° after 90-second equilibration period.

Fig. 2. Effect of enzyme concentration on reaction rate. The complete system contains: DDT, 0.24 μmole in 4.2 mg lipoprotein; GSH, 13 μmoles; phosphate 275 μmoles, pH 7.4; and DDT dehydrochlorinase as indicated. Reaction period 8.5 minutes, measured at 25° after 90-second equilibration.
500 μg. of neutralized Versene per ml. Glass distilled water was used throughout. Dialysis tubing was purified according to Klitz (9). DDT-resistant flies were reared by standard procedures (10) from eggs laid by parents exposed to a DDT residue. The flies were stored at -20° before processing for periods up to 4 months with minor losses in activity.

Preparation of Homogenate—Frozen flies in 50 gm. batches are homogenized for 1 minute in 200 ml. of 0.1 M sodium thiosulfate (recrystallized), pH 7.4 (0.02 M with respect to phosphate), at half speed in the Waring Blender. The speed is increased to full and maintained for an additional minute. The material is centrifuged for 5 minutes at 20,000 × g and the precipitate is discarded.

Fraction I—The supernatant liquid is brought to pH 5.0 by the addition of 2 M acetic acid and centrifuged as before. The orange-brown supernatant is decanted through cheesecloth to remove the fatty layer and brought to -3° in a stainless steel beaker placed in a controlled low temperature bath. A methanol solution precooled to -20° is added to 40 per cent (volume per volume) concentration while the temperature is permitted to drop to -15° (75 ml. of methanol per 100 ml. of enzyme preparation). The methanol contains 50 ml. 0.4 M sodium thiosulfate (adjusted to pH 5.0) per liter. The suspension is centrifuged at 4,000 × g for 5 minutes at -25° and the supernatant fluid is returned to the bath at -15°. The methanol concentration is raised to 65 per cent over a 45-minute period (150 ml. of additional methanol-thiosulfate per 100 ml. of original pH 5.0 enzyme preparation) at -15° to -19°. The material is centrifuged as before and the precipitate is washed rapidly at 0° with a volume of 0.05 M KH2PO4 equal to 20 per cent that of the original extract. The washings are removed by centrifugation at 0° and 5,000 × g and discarded. The precipitate is extracted with 0.02 M Na2CO3 for 4 hours (5 to 10 per cent of the original volume) and any insoluble material removed by centrifugation. The pink supernatant solution is dialyzed against 20 volumes (2 changes) of 0.05 M phosphate, pH 5.4, for 12 hours. The flocculent white precipitate is removed by centrifugation and the pale yellow supernatant solution is neutralized and dialyzed against 5 × 10^-4 M neutralized Versene and lyophilized.

Fraction II—A 1 per cent solution of the material from Fraction I is prepared in 0.1 M GSH (containing 0.01 M Versene) adjusted to pH 6.0. The material is transferred to a stainless steel beaker and brought to 45° with gentle stirring by immersion in a water bath maintained at 49-50°. After 4 minutes the preparation is cooled to 2° and centrifuged free from insoluble material at 20,000 × g. The supernatant solution is neutralized, dialyzed against 20 volumes of dilute Versene, pH 7.4, and lyophilized.

Fraction III—Amberlite IRC-50 (XE-64) is prepared according to Hirs (11). The 150 to 200 mesh material is used exclusively. The resin is cycled three times through 3 N H2PO4 and 3 N NH4OH. After the last alkaline pass the resin is washed with water until the washings are at pH 9.8 and transferred to the column. The dimensions of the column are 12 × 0.6 cm. for 10 to 12 units of enzyme (specific activity 0.6 to 0.8). The more dilute of the eluting buffers, 0.02 M ammonium phosphate is prepared by dissolving 6.6 gm. of (NH4)2HPO4 in 4.9 l. of water, adjusting to pH 5.85 with 15 N H2PO4 and making to a volume of 5 l. The more concentrated buffer, 0.50 M ammonium phosphate, is prepared in the same manner. The column is equilibrated with the 0.02 M buffer before chromatography.

About 10 units from Fraction II are dissolved in 3 ml. of 0.02 M buffer and dialyzed against the same buffer for 4 to 6 hours. The enzyme is placed on the column, followed by an additional 25 ml. of 0.02 M buffer. The column is transferred to a fraction collector for gradient elution. The mixing chamber should contain 110 ml. of the more dilute buffer, the reservoir 165 ml. of the 0.50 M buffer. Three-milliliter fractions are collected at a rate of 10 ml. per hour between the buffer concentrations of 0.24 and 0.36 M as determined by the equation of Alm et al. (12). Those fractions exhibiting an increase in purity greater than 1.6 times the material of Fraction II are combined, dialyzed against Versene, and lyophilized as before. The use of regenerated columns has not been successful.

Fraction IV—Calcium phosphate gel is prepared according to Kunitz (13) and aged at least 10 months. From 10 to 30 units of enzyme are dissolved in 20 ml. of water and brought to pH 6.0 with 0.2 M acetic acid. Any insoluble material is removed by centrifugation and gel (dry weight 33 mg. per ml.) is added to the material of Fraction II are combined, dialyzed against Versene, and lyophilized.

Fraction V—Ten to 15 units of enzyme are dissolved in 7 ml. of 0.1 M GSH (0.01 M Versene) at pH 5.0. The methanol thiosulfate procedure of Fraction I is followed and the 50 to 65 per cent fraction is collected. No thiomalic acid other than that in the methanol is present in the enzyme solution. After dialysis against 0.05 M phosphate (pH 5.4) and neutralization with 0.3 N NaOH the purified enzyme is held at 0° and used within 48 hours.

Remarks—The quantitative data concerning the fractionation are presented in Table II. The nonlinearity of the bead assay makes it difficult to assess the specific activities of the original fly extract in terms of the lipoprotein assay. The value of 0.0046 given in Table II represents a maximum for this term. Fly extracts have been prepared with barely measurable activity in terms of the bead assay which produced active material when

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Treatment</th>
<th>Total protein</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Supernate of homogenate</td>
<td>5000</td>
<td>23</td>
<td>0.00401</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>40 to 64% methanol</td>
<td>50</td>
<td>18.8</td>
<td>0.375</td>
<td>80</td>
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<tr>
<td>III</td>
<td>Flash heating</td>
<td>19</td>
<td>13.2</td>
<td>0.705</td>
<td>57</td>
</tr>
<tr>
<td>IV</td>
<td>Chromatography</td>
<td>6.6</td>
<td>8.6</td>
<td>1.30</td>
<td>37</td>
</tr>
<tr>
<td>V</td>
<td>Calcium phosphate gel</td>
<td>3.0</td>
<td>6.5</td>
<td>2.12</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>5 to 65% methanol</td>
<td>0.25</td>
<td>0.65</td>
<td>2.60</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* Specific activity of lipoprotein assay = AE280/mg. protein/10 minutes.

† Estimated from the specific activity of bead assay = 0.014 mmole DDE/mg. protein/90 minutes.
subjected to Step I of the fractionation scheme. In these instances the purification index required to achieve a specific activity of 2.6 was 4- to 8-fold greater than those encountered with more active preparations. This high degree of purification necessary is probably the result of the use of an entire multicellular animal rather than a specific organ as the starting material. Ammonium sulfate fractions of fly extracts may be prepared which show a specific activity of about 0.03. The enzyme precipitates at 0.9 to 0.75 saturation at this stage, and neither the rejected ammonium sulfate nor the 0 to 40 per cent methanol fractions inhibit the enzyme. After Step II each lyophilization during the fractionation scheme is accompanied by a 20 per cent loss in activity. A 20 per cent loss is sustained during dialysis of preparations of material of specific activity >0.4. Storage of 1 per cent solutions in the freezer at this purity level results in 50 per cent inactivation. The addition of GSH, KCl, glycine, or histidine buffers does not overcome losses in activity during dialysis, lyophilization, or freezing. No improvement in yield was obtained by fractionation procedures involving sorption on activated charcoal, Zn(OH)₂, or alumina gel C₄. Fractionation with protamine sulfate, nucleic acid, acetone, dioxane, or ethanol with or without the addition of Zn²⁺, Mn²⁺, or Mo⁶⁺ met with no success. Salt fractionation with dibasic ammonium phosphate or neutral or acid ammonium sulfate is to be avoided after Step II; only 50 per cent of the activity could be recovered at 0.95 saturation with these salts. Selective denaturation with Ag⁺ met with only limited success. The effort involved in the rearing of resistant flies and the low yield and instability of the purified enzyme placed an obvious limit on the extent of investigations with material of high purity.

Physical and Chemical Properties of DDT Dehydrochlorinase

**Purity**—Further fractionation of material of specific activity 2.6 with calcium phosphate or alumina gel C₄, ammonium sulfate, or methanol fails to yield material of greater purity. Ultracentrifugal analysis with the ultraviolet absorption optical system shows a single component with an extinction coefficient value of 3.6 × 10⁻¹³ second. Assuming an unhydrated sphere with a specific volume of 0.75, the molecular weight is calculated as 36,000. The instability of the enzyme to freezing and thawing and to organic solvents at temperatures above 5 degrees suggested the presence of lipide. Ultracentrifugal flotation in 10 per cent NaCl or 25 per cent KBr at 30,000 X g with material of specific activity 0.7 failed to reveal any activity in the protein fractions of low density. Highly purified material could not be prepared in sufficient quantity for electrophoretic analysis. The spectrum of the purified enzyme is that of a simple protein, E₁底气₅₀ (278 μm) is 9.8 in phosphate buffer, pH 7.4. Material prepared from flies stored for long periods has a yellow-brown appearance even at a specific activity of 2.6 probably due to oxidation of tyrosine residues by tyrosinase in the frozen flies (14). The ratio 280/260 μm is 1.6 for the purified enzyme indicating little or no contaminating nucleic acid or bound nucleotides. At specific activity 2.0 no tyrosinase, GSH reductase, acetylcholine esterase, or triosephosphate dehydrogenase activity can be detected.

**Optimum pH and Isoelectric Point**

The optimal pH for DDT dehydrochlorinase is 7.4. Fig. 3 shows that the active hydrogen on the tertiary carbon of DDT ionizes little if any in the lipoprotein system, for the usual stimulation accompanying proton-producing reactions is not evident at alkaline pH. No nonenzymatic dehydrochlorination could be observed at pH 0.5. The meaning of the shoulder at pH 7.5 to 8.5 is, at present, obscure. No significant differences in rate could be observed in phosphate, pyrophosphate, tris(hydroxymethyl)aminomethane, Versene, borate, imidazole, acetylde, acetate, or glycylglycine buffer at the relatively high ionic strength required to maintain solution of the lipoprotein. The enzyme is stable for 6 hours in the pH ranges 4 to 6 and 10 to 12 at 2° for 48 hours at pH 0 to 6.10. The isoelectric point is in the vicinity of pH 6.5, a 0.5 per cent solution of the purified enzyme after dialysis against water for 48 hours exhibits a pH of 6.6, but with a 40 per cent loss in activity. The enzyme is completely soluble in water or dilute salt solutions at 2 per cent concentration in the pH range 4 to 12.

**Effect of Temperature**

Except under the conditions of the flash heating step in the fractionation scheme, temperatures above 45° cannot be maintained for periods greater than 10 minutes without almost complete loss of activity. Addition of the water-soluble sodium salt of the DDT analogue, 2,2-bis-(p-chlorophenyl)acetic acid in place of GSH in Step II does not render the enzyme stable to heating.

**Kinetics**—As measured between 20 and 30° with the lipoprotein assay, AH of the enzyme-catalyzed reaction is 20.5 kilocalories per mole. Cristol et al. (15) report a value of 18.6 kilocalories per mole for the base-catalyzed dehydrochlorination in this temperature range. The lipoprotein assay also permits an evaluation of the equilibrium of the reaction. In common with most E₂-type reactions, equilibrium lies far to the right. No unchanged DDT can be detected after 60 minutes at 30° by colorimetric or spectrophotometric analysis of a reaction mixture containing 2 μmoles of DDT and 10 units of enzyme after chromatography on alumina by the procedures of Sternburg and Kears (2, 16, 17). These procedures can detect 2 × 10⁻⁸ μmoles of DDT. These data are supported by studies of the time course of DDT dehydrochlorinase action (Fig. 4) and indicate that Kₑ₅₀ > 1,000, well beyond the range permitting any accurate determination of ΔF.

The Michaelis-Menten constants have been determined with
purified enzyme, since the lipoprotein assay makes possible the measurement of initial rates. The constant for DDT, \( K_{\text{nD}} \) is 5 \( \times 10^{-7} \) mole per liter. The unusually high levels of the activator required by the enzyme are evident from the value of the constant for GSH \( K_{\text{nG}} \) which is 2.5 \( \times 10^{-4} \) mole per liter. Attempts to analyze the kinetic data according to the formulas devised for two-substrate systems by Frieden (18) and Florini and Vestling (19) do not clarify the mechanism of the reaction, since a positive point of common intersection was observed when 1/\( v \) versus 1/DDT was plotted at different concentrations of GSH. The lag period preceding the attainment of a constant rate of optical density change (see assay) is of the same duration regardless of the order in which enzyme, substrate, and cofactor are added to the system. With DDT as substrate, and GSH alone as cofactor, the turnover number is 2.3 moles of DDT per mole of enzyme per minute at 25°. When measured at 35° with 1,1-dichloro-2,2-bis-(p-chlorophenyl)ethane as substrate and a mixture of GSH and cysteinyglycine as cofactor this value is increased approximately 30-fold (4).

Isolation of Enzyme from DDT-susceptible Flies—The development of resistance to DDT over the last decade in populations of houseflies more or less completely susceptible to this insecticide has resulted in much speculation regarding the origin of this phenomenon (20, 21). Attempts to induce the development of the DDT-detoxifying system with sublethal doses of the insecticide have met with uniform lack of success (22, 23). Sternburg et al. (2) failed to detect any DDTase in susceptible strains with the use of homogenates in conjunction with the glass bead assay, thus the possibility of selection of a few DDTase-positive individuals in a large population by the insecticide was discounted. The development of the sensitive lipoprotein assay and a scheme for purifying the enzyme have made a reinvestigation of this question possible. By the methods described, 25,000 susceptible (CSMA strain) flies were processed through Fraction V and assayed. About 0.003 unit of enzyme was detected in the final preparation, or 0.05 per cent of that present in an equivalent number of resistant individuals. The enzyme from susceptible flies is similar in specificity to the enzyme in resistant individuals (4). The exact magnitude of this figure is of minor consequence, but it is apparent that the enzyme seems to occur in susceptible populations.6

DISCUSSION

The observation that the equilibrium of reaction lies far to the right when DDT is presented to the enzyme in lipoprotein is of considerable significance when considering the survival of flies exposed to DDT. It is generally accepted that the site of DDT action is the nervous system (24), a tissue rich in lipides and lipoproteins. This tissue contains high titers of DDT dehydrochlorinase as well (25), and it is probable that this juxtaposition of detoxifying enzyme and DDT-solubilizing substances results in a rapid and complete degradation of toxin reaching the sensitive target. The finding that measurable amounts of enzyme can be identified in susceptible populations may indicate that the enzyme mediates some other reaction as well as the elimination type, is of common occurrence in living systems. The isolation of the enzyme from susceptible flies lends biochemical evidence to the report by Kerr and Venable (26) that DDT dehydrochlorinase may be identified in populations of flies not exposed to DDT but selected for late emergence from the pupal stage of development. Before a search for auxiliary substrates of natural origin can be undertaken, however, problems of instability and low yield must be overcome.

SUMMARY

1. A method for the isolation of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT)-dehydrochlorinase from DDT-resistant flies has been described and some of the properties of the purified enzyme are reported.

2. A rapid spectrophotometric assay has been devised with lipoprotein-solubilized DDT as substrate.

3. It is shown that a small amount of enzyme occurs in populations of susceptible flies and the implication of these and the kinetic data are discussed with respect to the nature and origin of resistance to DDT.

Acknowledgments—Dr. B. Roger Ray, now at Washington State College, suggested lipovitellin as a convenient source of lipoprotein and provided generously of his time and knowledge concerning its preparation. Dr. K. E. VanHolde executed the ultracentrifugal analysis, and Drs. E. D. Ihaen and T. Huang made several attempts at electrophoresis of dilute enzyme preparations.

REFERENCES


6 The presence of this enzyme in susceptible housefly larvae has recently been demonstrated by Dr. Herbert Moorefield of the Boyce Thompson Institute (personal communication).
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