DDT Dehydrochlorinase

II. SUBSTRATE AND COFACTOR SPECIFICITY*

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In Paper I (1) of this series methods of purification and measurement of DDT dehydrochlorinase† were described. Those physical and chemical properties that could be determined with the small amounts of purified enzyme available were presented. It was shown that proton liberation from the tertiary carbon atom of DDT (C-2) was not stimulated at alkaline pH although the enzyme possesses considerable stability between pH 7 and 10. Differences in specificity previously reported (2) as well as the depressed rate of reaction at high pH distinguishes the enzymic mechanism from the base-catalyzed elimination described by Cristol et al. (3). For the purpose of clarifying the detoxifying mechanism a study has been made of the effect of alternative substrates, cofactors, and group-specific inhibitors on the reaction rate.

Sternburg et al. (2) with the use of crude enzyme preparations and the bead assay showed that only those halogenated hydrocarbons whose structure resembled DDT were attacked, and that the p,p'-ring substituents were obligatory. The turnover of GSH during the reaction was followed by both the titrimetric method of Bray et al. (4), and the nitroprusside reaction. No oxidation of the SH group could be detected. The inability of the enzyme to attack the small amounts of purified enzyme available were presented. With respect to cofactor specificity, both the DDT and TDE systems are stimulated by the addition of cysteinyl-glycine. Additional evidence indicating the identity of the enzyme degrading DDT and TDE is presented in Fig. 1 where the inhibition of the TDE reaction by DDT is presented. With respect to cofactor specificity, both the DDT and TDE systems are stimulated by the addition of cysteinyl-glycine.

Identification of TDE Reaction Product—The compound TDE may form two elimination products, namely the 1-chloroethylene or dehydrochlorination, or the 1,1-dichloroethylene (DDE) by dehydrogenation. DDT yields only the latter compound (2). The high reaction rate with TDE might indicate that DDTase is in reality a dehydrogenase of low specificity, rather than a dehydrochlorinase, a supposition that would do much to explain the occurrence of this enzyme in susceptible populations. The importance of this thesis made necessary the identification of the reaction product with TDE. A reaction system containing 30 moles of TDE in lipoprotein was incubated until the reaction had achieved completion. The solution was deproteinized, and extracted with petroleum ether. The ether was washed with 1.0 per cent NaOH and water and chromatographed on alumina (6). The petroleum ether eluate from the column was rechromatographed and examined in the spectrophotometer. Fig. 2 shows that the reaction product has the spectral characteristics of the

RESULTS

Effect of Substrate Structure on Reaction Velocity—Table I shows that the initial rates of the 2,2-bin(p-Cl) and -(p-Br) analogues of DDT are the same in the lipoprotein system. Of the other p,p'-substituents tested, p-F-, CH-, CH,O-, and I-react at lesser rates and the unsubstituted rings not at all. The most noteworthy feature of the rate with alkyl-substituted derivatives of DDT is the high rate of dehydrochlorination of the 1,1-dichloroethane derivative (TDE), almost 4 times the rate of the reference compound, DDT. The 1-chloro derivative also reacts at a high rate although product inhibition occurs with this and the unchlorinated alkyl derivative.

The strain of flies used in the preparation of the enzyme has been exposed to no insecticide other than DDT. In Paper I (1) the isolation of a small amount of enzyme from DDT-susceptible flies was reported. This preparation also attacks TDE at a higher rate than DDT. With extracts of resistant flies the ratio of the rate of dehydrochlorination of TDE to DDT remains at 3.8:1 through all the stages of purity that can be assayed in the lipoprotein system. Sternburg et al. (2) reported a ratio of about 0.5 with the use of the bead assay with 4 mg. of substrate per vessel. Reinvestigation of this observation with equimolar quantities of substrate, in the bead assay show that higher rates of dehydrochlorination are obtained with TDE just as in the case with the lipoprotein system. Additional evidence indicating the identity of the enzyme degrading DDT and TDE is presented in Fig. 1 where the inhibition of the TDE reaction by DDT is presented. With respect to cofactor specificity, both the DDT and TDE systems are stimulated by the addition of cysteinyl-glycine.

Activity determinations were performed with lipoprotein or on glass beads by methods previously described (1, 2). Molecular extinctions of the various DDT analogues and their olefins were measured in ethanolic solutions or taken from Sternburg (2). These compounds have been synthesized in this laboratory over the course of several years or obtained commercially in reality a dehydrogenase of low specificity, rather than a dehydrochlorinase, a supposition that would do much to explain the occurrence of this enzyme in susceptible populations. The importance of this thesis made necessary the identification of the reaction product with TDE. A reaction system containing 30 moles of TDE in lipoprotein was incubated until the reaction had achieved completion. The solution was deproteinized, and extracted with petroleum ether. The ether was washed with 1.0 per cent NaOH and water and chromatographed on alumina (6). The petroleum ether eluate from the column was rechromatographed and examined in the spectrophotometer. Fig. 2 shows that the reaction product has the spectral characteristics of the

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‡ The abbreviations used are: DDT, 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; TDE, 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethane; BAL, 2,3-dithiopropanol.
TABLE I
Dehydrochlorination of analogues of DDT by DDT Dehydrochlorinase
The system contained at least 0.3 pmole of substrate in 4 mg. lipoprotein; GSH, 13 pmoles; phosphate, 275 pmoles, pH 7.4. Reaction time 10 minutes, at 25°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Olefin formed (μmoles x 10⁻⁸)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ring substituted</td>
<td></td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane</td>
<td>21.1</td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(p-bromophenyl)ethane</td>
<td>21.2</td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(p-fluorophenyl)ethane</td>
<td>11.3</td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(p-tolyl)ethane</td>
<td>7.6</td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(p-methoxyphenyl)ethane</td>
<td>4.9</td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(p-iodophenyl)ethane</td>
<td>1.8</td>
</tr>
<tr>
<td>1,1,1-Trichloro-2,2-bis(phenyl)ethane</td>
<td>0.0</td>
</tr>
<tr>
<td>Alkyl substituted</td>
<td></td>
</tr>
<tr>
<td>1,1-Dichloro-2,2-bis(p-chlorophenyl)ethane (TDE)</td>
<td>79.9</td>
</tr>
<tr>
<td>1-Chloro-2,2-bis(p-chlorophenyl)ethane</td>
<td>25.2*</td>
</tr>
<tr>
<td>2,2-Bis(p-chlorophenyl)ethane</td>
<td>0.6*</td>
</tr>
</tbody>
</table>

* Initial rate maintained only 3 minutes.

Effect of Group-specific Inhibitors—The observation by Sternburg et al. (2) that activation of the enzyme could not be achieved by cysteine or BAL indicated that the role of GSH in the system was not that of a simple reduction of enzyme SH groups. This has been verified by a study of the effect of SH-binding agents by the procedure of Edwards and Knox (7) in which the rate is measured in the presence of marginal levels of GSH. Since any inhibition that occurs can be due to either a reduction in GSH concentration or to a blocking of enzyme SH, the subsequent addition of GSH in excess can be used to differentiate between the two effects. Table II shows that N-ethylmaleimide, cupric, mercuric, arsenite, or silver ion has no effect on the system. Iodoacetate inhibits to a minor degree and the inhibition is almost completely reversible. The inhibition observed with p-chloromercuribenzoate appears to be a property of the p-HgCl moiety rather than the SH binding capacity of this compound as the inhibition is partially reversible. Moreover, the studies of Moorefield and Kearns (5) demonstrate the adverse effect of p-substituted ring compounds on the reaction rate.

The insensitivity of the enzyme to metal binding agents is also described in Table II. High concentrations of cyanide, azide, α,α-dipryridyl, and 8-hydroxyquinoline are without effect. Cysteine does not compete with GSH for the active site and fluoride and arsenate fail to inhibit.

Effect of Miscellaneous Cofactors and Metals on Reaction Rate—The effect of prolonged dialysis against water was studied with enzyme of specific activity 0.7 which exhibits greater stability than more refined material. As assayed with citrate-dialyzed lipoprotein, no loss in activity was evident after dialysis for 26 hours against 3 changes of water. The inclusion of Versene (ethylenediaminetetraacetate) in the assay system at 10⁻⁴ M does not inhibit the enzyme. At higher purities considerable activity is lost during dialysis against water unless Versene is present.

The purified enzyme is not stimulated by the addition of Al³⁺, Mn²⁺, Cu²⁺, Fe²⁺, Zn²⁺, Mg²⁺, or Ca²⁺ at 10⁻⁴ M.

As measured with enzyme at specific activity 2.0 the addition of DPN at 10⁻⁴ M is without effect on the reaction rate. No change in optical density at 340 μm was observed. The addition of FAD at 10⁻⁴ M or pyridoxal phosphate at 10⁻⁵ M does not
stimulate the enzyme. None of the cofactors or metals listed above exert any inhibitory action.

Effect of Sulphydryl Compounds on Reaction Rate—The specificity of SH compounds was determined by observing the reaction rate after the addition of 10 μmoles of the activator to the system containing enzyme (specific activity 1.5) and lipoprotein-DDT. Compounds with high absorbance at 260 nm were tested by colorimetric measurement of DDE after chromatography on alumina (6). The possible ability of SH compounds to inhibit the dehydrochlorination reaction was determined by the subsequent addition of 10 μmoles of GSH to the system.

No dehydrochlorination could be detected with cysteine, BAL, ethanol mercaptide, homocysteine lactone, thiolhistidine, ergothioneine, thioglycollate, thialmate, CoA, oxidised glutathione, S-acetyl glutathione, ascorbate, or glycylglycine. These compounds do not stimulate or inhibit the reaction when GSH is added to the system. Preincubation of the enzyme with 1 μmole of ascorbate or GSH for 30 minutes at 25° does not reduce the level of GSH subsequently required for the achievement of the maximal reaction rate. Glutamyl-cysteine is unable to serve as cofactor for the enzyme, but unlike cysteine, the dipeptide inhibits the enzyme when added on a mole for mole basis with GSH. Glutamyl-cysteine is not a potent inhibitor, as shown in Fig. 3; lesser amounts than 10 μmoles are without appreciable influence on the reaction rate. With cysteinyl-glycine as cofactor, however, the addition of glutamyl-cysteine brings about an abrupt termination of the reaction (Fig. 4).

The ability of cysteinyl-glycine to activate the reaction is shown in Fig. 4. The addition of 5 μmoles of cysteinyl-glycine to a system lacking in GSH maintains a rate about 60 per cent of an equivalent amount of GSH. Levels of cysteinyl-glycine greater than 5 μmoles do not lead to further stimulation of the reaction. In the presence of an excess of GSH (10 μmoles) only catalytic quantities of cysteinyl-glycine are required to effect a 50 per cent stimulation, the rate when both compounds are present being greater than that observed with either material alone.
(Fig. 4). The action of this dipeptide can be observed equally well if added prior to the addition of GSH or at the same time (Fig. 4). The effect of cysteinyl-glycine additions to systems containing GSH is observable with TDE as substrate as well as with DDT.

**DISCUSSION**

The stability of DDT dehydrochlorinase to SH-binding reagents and the high order of specificity with respect to co-factor requirements are somewhat unique, the indications being that the active site does not involve enzyme-SH. A study of the lag period before the attainment of a constant rate of reaction fails to show any preliminary formation of a bimolecular complex such as enzyme-GSH or enzyme-DDT, as measured by a decrease in the lag period. Such complexing may be very rapid or complex formation may be envisioned as trimolecular ignoring the possible participation of the elements of water. A search for spectrophotometric intermediates in the range 260 to 425 nm gave essentially negative results. At shorter wave lengths, where absorption from transient thioethers might be predicted (8) no measurements were possible as a consequence of the end absorption of the lipoprotein carrier. The failure to observe any oxidation of GSH during the course of reaction may indicate, on the other hand, that the participation of the cysteine moiety of GSH in the reaction is not direct, regardless of its essential role in the reaction. The possible regeneration of GSH during the dehydrochlorination or the involvement of the glycine residue of GSH constitute additional possibilities.

An intriguing similarity of properties and function exist between DDT dehydrochlorinase and the nitroglycerin reductase of pig liver investigated by Heppel and Hilmoe (9). Not only are the solubility characteristics and adsorbability of the two systems on calcium phosphate gel almost identical, but also both systems respond to GSH and to a lesser degree to cysteinyl-glycine. Perhaps the most challenging coincidence with respect to the liver and fly enzymes involves their common detoxicating function and the absence of known substrates of natural origin. In the case of DDT dehydrochlorinase the demonstration that the reaction in the presence of GSH is stimulated by catalytic levels of cysteinylglycine suggests that the relatively low turnover number of the enzyme in vitro may be increased in situ by still other materials which could serve to further increase the reaction rate.

**SUMMARY**

1. The specificity of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) dehydrochlorinase for various analogues of DDT has been investigated. The p-Br compound is degraded at the same rate and the 1,1-dichloroethane derivative approximately 4 times as fast as DDT.
2. The enzyme shows little or no sensitivity to SH inhibitors or metal-binding agents
3. Of the compounds tested, only glutathione and cysteinylglycine will initiate the reaction. The addition of catalytic quantities of the dipeptide to the glutathione-fortified system stimulates the dehydrochlorination reaction.
4. Glutamyl-cysteine is devoid of activity as a co-factor, and shows some inhibitory action.

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**REFERENCES**
