Note

Dehydrochlorination Mechanism of DDT Analogs Catalyzed by Housefly Enzyme

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One of us¹⁾ recently obtained two apparently homogeneous enzymes from the housefly that dehydrochlorinate DDT (Fig. 1, X=Cl, Y=H) in the presence of glutathione (GSH). Each enzyme also conjugates GSH with 2,4-dichloronitrobenzene and 1-chloro-2,4-dinitrobenzene (CDNB).

As a first step towards elucidating the DDT dehydrochlorination mechanism we conducted kinetic studies on a housefly-enzyme preparation purified from the Victoria A strain. Decapitated Victoria A strain houseflies (150 g) were homogenized in 300 ml of 0.01 M phosphate buffer, pH 7.4 (1 mM cysteine added), and the mixture was centrifuged at 10,000 $\times g$. The supernatant was put on an affinity column of bromosulphophthalein (BSP)-glutathione conjugate immobilized on Sepharose 4B, and the loaded column was washed with 5 volumes of 0.5 M KCl solution.

The enzyme was eluted with 5 mM BSP (75 ml), a total of 125 ml of enzymatically active fractions being collected and pooled. Part of the pooled material (60 ml) was chromatographed on a Sephacryl S-200 column (5 $\times$ 40 cm) in 0.025 M Tris acetate buffer, pH 8.3, fractions that were enzymatically active being collected and pooled (110 ml). The enzyme solution contained 194 $\mu\text{g/ml}$ of

protein with GSH *S*-transferase activity on CDNB of 8.34 $\mu\text{mol/min/mg}$ protein (1 mM CDNB, 1 mM GSH, pH 6.5, 25°C) and DDT dehydrochlorinase activity of 2.35~2.74 nmol/min/mg protein (2 μM DDT, 1 mM GSH, pH 7.4, 37°C). The frozen enzyme solution was packed in dry-ice, air-mailed from Wellington to Kyoto, and stored at -70°C until used.

The substrates used were DDT analogs that had various substituents in place of the two aromatic chlorines of DDT. For the isotope effect study, methine-deuteriated DDT analogs (Fig. 1, Y=D) were prepared and used. These analogs were prepared by published methods^{2~4)} with slight modifications. LiAlD₄ or NaBD₄ (D > 98%) reduction of 4-chlorophenyl trichloromethyl ketone (tetrachloroacetophenone) to a methine-deuteriated carbinol that was then condensed with chlorobenzene gave deuterio-DDT (Fig. 1, X=Cl, Y=D) from tetrachloroacetophenone in an 80% yield.

Ketone precursors for the methoxy and methyl analogs (Fig. 1, X=CH₃O or CH₃, Y=D) were prepared by the Hoesch-Houben reaction.⁵⁾ The nitro (NO₂) analog with methine-deuterium was prepared by nitration of the unsubstituted analog (Fig. 1, X=H, Y=D). All the deuteriated analogs used were purified by repeated recrystallization. Melting points and microanalytical data are given in Table I.

The enzymatic reaction mixture (2 ml) consisted of the substrate (at one of several amounts between 0.2 and 100 nmol in 20 μl dimethyl sulfoxide), GSH (2 μmol , or an amount between 0.2 and 4.0 μmol for the study of the rate-GSH concentration relation), enzyme (20 to 40 μl : 3.88 to 7.76 μg protein as assayed by an improved Lowry method⁶⁾) and 0.1 M sodium phosphate of pH 7.4. The incubation temperature was 37°C.

FIG. 1. Structure of the DDT Analogs Used as Substrates.

X=OCH₃, CH₃, H, Cl, and NO₂. Y=H and D.

TABLE I. MELTING POINTS OF AND ANALYTICAL DATA ON METHINE-DEUTERIATED DDT ANALOGS

Structure ^a Substituent X	Melting point (°C)	Analytical data (%)			
		Found C	H & D	Calcd. C	H & D
CH ₃ O	87.5	55.56	4.48	55.44	4.65
CH ₃	90.5	60.87	4.91	61.07	5.12
H	64	58.73	4.08	58.67	4.22
Cl	108.5	47.38	2.71	47.30	2.83
NO ₂	173	44.86	2.42	44.65	2.68

^a The chemical structure is shown in Fig. 1, in which Y is deuterium and X is the substituent given in this table.

TABLE II. RATE PARAMETERS FOR ENZYME-CATALYZED DEHYDROCHLORINATION OF DDT ANALOGS^a

Substituent (X)		CH ₃ O	CH ₃	H	Cl	NO ₂
$V_{\max}^b$	methine Y = H	0.741	0.746	1.50	4.81	141.2
	Y = D	0.0875	0.105	0.169	0.762	42.7
$^dV^c$		8.47	7.10	8.88	6.31	3.31
σ_p^d		-0.268	-0.170	0.000	+0.227	+0.778

^a Reaction conditions: GSH 1 mM in 0.1 M sodium phosphate pH 7.4 at 37°C.

^b $V_{\max}$ is expressed in nmol/min/mg protein. Derived from the rate data at high substrate concentrations (see the text for details).

^c dV is a conventional expression of $(V_{\max})_H/(V_{\max})_D$; i.e., the apparent isotope effect on $V_{\max}$.

^d According to Hammett.⁷⁾

After a specified period, the substrate and metabolite (1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene (DDE) or one of its analogs) were extracted with hexane-ethyl acetate (4:1, v/v). The substrate and metabolite were measured by high-performance liquid chromatography (HPLC) for the NO₂ analog or by gas chromatography (GC) with an electron-capture detector for the other substrates. The HPLC solvent was dioxane-methanol-water (3:4:3, v/v), the column a 20-cm M&S Pack C18, and the detector UV254. GC column packings and temperatures for a column length of 75 cm were 1.5% NPGS, 220°C for CH₃O; 1% OV-17, 180°C for CH₃; 1% OV-17, 160°C for the unsubstituted and 1% OV-17, 200°C for the Cl analog.

For all the substrates, Lineweaver-Burk plots gave a biphasic curve with a gentler slope at high concentrations (Fig. 2). As the K_m and $V_{\max}/K_m$ could not, therefore, be calculated unequivocally, we only calculated the $V_{\max}$ values from extrapolations of data for the higher substrate concentrations. These $V_{\max}$ values and the ratio of the $V_{\max}$ of the protio-analog and the $V_{\max}$ of the deuterio-analog (i.e., the apparent deuterium isotope effects on $V_{\max}$) are listed in Table II along with Hammett's σ_p values for the substituents.⁷⁾ The reaction rates also depended on the GSH concentration; the results for one substrate (X = Cl, Y = H) are shown in Fig. 3. Strictly speaking, the $V_{\max}$ values in Table II should have been extrapolated to the value for an infinite GSH concentration, but the values given are sufficient for this discussion.

The biphasic nature of the double reciprocal plots might be the result of changes in the preferred reaction route in a supposed random sequential mechanism between two concentration ranges of DDT analogs.⁸⁾ The observed isotope effect value for the NO₂ analog was much smaller than the theoretical maximum and, as yet, there is no data from which to find whether this small value is close to the intrinsic value and reflects a chemical mechanism such as a more typical E1cB, or whether it is a suppressed value caused by relatively slow enzyme-product (EP) dissociation and so is much smaller than the intrinsic value. (For a detailed discussion of the suppression of the intrinsic isotope effect to a smaller value by a slow EP

FIG. 2. Lineweaver-Burk Plot of the Enzyme-Catalyzed Dehydrochlorination of DDT.

The reaction mixture (2 ml) consisted of 3.9 μg protein of enzyme, 2 μmol of glutathione, one of the specified concentrations of *S* (substrate DDT, Fig. 1, X = Cl, Y = H, in dimethyl sulfoxide) and 0.1 M sodium phosphate, pH 7.4. Incubation at 37°C.

FIG. 3. Lineweaver-Burk Plot of the Enzyme-Catalyzed Dehydrochlorination of DDT for Different Concentrations of Glutathione.

The reaction mixture (2 ml) consisted of 7.8 μg protein of enzyme, one of the specified concentrations (0.1, 0.2, and 0.4 μM) of *S* (substrate DDT, Fig. 1, X = Cl, Y = H), one of the specified concentrations of glutathione, and 0.1 M sodium phosphate, pH 7.4. Incubation at 37°C.

dissociation step, etc., see Northrop.⁹⁾ The latter possibility cannot be excluded because the very fast reaction rates of the NO₂ analogs, especially of the [methine-

FIG. 4. Proposed Reaction Mechanism for Enzyme-catalyzed Dehydrochlorination of DDT in the Presence of Glutathione (GSH).

The H or D to be removed would be situated midway between the attacking sulfur of GSH and the methine-carbon of DDT, thereby forming a highly symmetrical S---H(or D)---C arrangement in the transition state. Ar = aryl group; *p*-chlorophenyl group.

$^1\text{H}/\text{NO}_2$ analog, can make the EP dissociation step relatively slow.

A Hammett plot of $\log V_{\max}$ against σ_p that is based on the results given in Table II is moderately linear, the general trend being for $\log V_{\max}$ to increase with an increasing σ_p value as is obvious in both the ^1H and $^2\text{H}(\text{D})$ -series. The positive and highly significant ($r \doteq 0.99$) correlation between $\log V_{\max}$ and σ_p indicates an initial rate-limiting attack of a base (or anionic species) on the methine hydrogen followed by eventual HCl elimination.

The kinetic deuterium isotope effect values for the four

analog ($\text{X} = \text{CH}_3\text{O}$, CH_3 , H, and Cl) were large enough for us to assume the absence of heavy suppression.⁹⁾ These large values indicate a highly symmetrical transition state (Fig. 4) and are strong evidence for an E2 or irreversible E1cB mechanism.

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