SYNTHESIS OF [methine-^{3}H]DDT AND ITS NITRO-ANALOG, AND ISOTOPE EFFECTS IN THEIR ENZYME-CATALYZED DEHYDROCHLORINATION

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SUMMARY

[methine-^{3}H]1,1-Di-(4-chlorophenyl)-2,2,2-trichloroethane ([methine-^{3}H]DDT) and its di-(4-nitrophenyl) analog, both of high purity with a moderately high specific activity were prepared. Chlorobenzene was condensed with [1-^{3}H]1-(4-chlorophenyl)-2,2,2-trichloroethanol, which had been synthesized by sodium boro[^{3}H]hydride reduction of 4-chlorophenyl trichloromethyl ketone. The purified [^{3}H]DDT had a specific activity of 0.77 Ci/mmol (28.49 GBq/mmol). [methine-^{3}H]1,1-Diphenyl-2,2,2-trichloroethane was similarly synthesized and was nitrated to give [methine-^{3}H]1,1-di-(4-nitrophenyl)-2,2,2-trichloroethane of 1.63 Ci/mmol(60.31 GBq/mmol). Dehydrochlorination with housefly enzyme (glutathione-dependent DDT dehydrochlorinase) showed a remarkable isotope effect. For DDT, the observed tritium isotope effect on V_{max}/K_m was 11.51±0.52. For the nitro-analog, the value was 11.3±1.2. We measured deuterium isotope effect on V_{max}/K_m for DDT in a competitive mode and obtained the value 4.19±0.34. Based on these values, the magnitude of intrinsic isotope effect values on DDT-dehydrochlorination reaction was discussed.

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INTRODUCTION

Hydrogen isotope effects on an enzyme-catalyzed reaction give us useful information for elucidating the reaction mechanism involving C-H bond cleavage. But simply observed isotope effect values are often masked by slow steps involved in enzyme-catalyzed reactions, such as ES-complex formation and dissociation and EP-complex dissociation steps(1). Such masked values are often much smaller than the intrinsic value and do not allow us their straightforward use to elucidate mechanism. One of the procedures to overcome this difficulty is to calculate an intrinsic isotope effect value from observed values of deuterium and tritium isotope effect on the same reaction(1). For doing this, we need a tritium-labeled substrate of high purity with a moderately high specific activity.

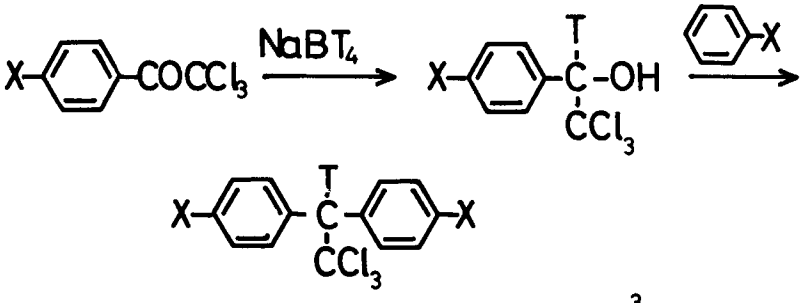
A few years ago, a housefly enzyme that catalyzes DDT-dehydrochlorination was purified (2). With this enzyme preparation, we conducted a deuterium isotope effect study (3) on DDT analogs in a non-competitive mode (1). Here we describe the synthesis of two of the methine-tritiated analogs, and their use in isotope effect studies on the dehydrochlorination catalyzed by the housefly enzyme. We also describe the measurement of deuterium isotope effect in competitive-type experiments and use of these isotope effect values for calculating the intrinsic isotope effect on the metabolic dehydrochlorination.

SYNTHESIS

Materials and Methods Sodium boro[^{3}H]hydride and [phenyl-U-^{14}C]DDT were obtained from Amersham International plc. Trichloroacetophenone (1-phenyl-2,2,2-trichloroethanone) was prepared from acetophenone by the method of McLennan and Wong (4) with a slight modification (3). [methine-^{2}H]DDT (D-abundance >99.5%) was prepared as described (3). Precoated thin layer chromatographic silica gel plates were obtained from Merck ($60F_{254}$) and used with a solvent system hexane/acetone 10/1 (v/v) for analysis and purification. For radioactive spot-detection, a beta-camera(Berthold) or a position-sensitive proportional detector (Aloka chromanizer) was used. Liquid scintillation (LS) counters were Aloka LS-900 and LS-1000 and the

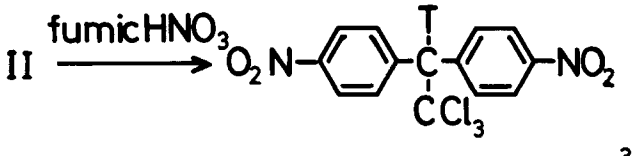
scintillation cocktail was Aquasol-II. Gas chromatograph (GC) was a Yanaco G-80E with an electron capture detector(^{63}Ni) (column:1% OV-17, 75 cm), and high performance liquid chromatographs (HPLC) were a Waters with model 60004 pump and model 440 detector, and a Jasco with 880-PU(pump) and 875-UV(detector). They were used with a column of M&S Pack C_{18}, 4.0 mm I.D. x 250 mm and a solvent system dioxane/methanol/water, 3/5/2 (v/v). Radioactivity determinations of HPLC effluent were conducted by collecting fractions and LS-counting of each fraction.

Reduction of 4-chlorophenyl trichloromethyl ketone with sodium boro-[^{3}H]hydride(Scheme 1) Sodium boro[^{3}H]hydride (ca.100 mCi, 3.7 GBq)(5-10



(I) X=Cl ([methine-^{3}H]DDT)
(II) X=H

Scheme 1 Synthesis of [methine-^{3}H]DDT and its analog



Scheme 2 Synthesis of [methine-^{3}H]NO_2-DDT

Ci/mmol, 185-370 GBq/mmol) (20-10 μmol) was diluted with $NaBH_4$ (380 μg, ca.10 μmol) in 0.01 N NaOH (0.3 ml) and was mixed with the ketone (40 mg) in isopropyl alcohol (3 ml) while stirring at 0°C. Stirring was continued for 4.5 hours. After acidification with 2 N HCl and dilution with water,

the mixture was shaken with ether (3 ml x 3). The combined ether solution (9 ml) was evaporated and the residue was chromatographed on TLC(See Materials and Methods for the condition). Radioactive area on the TLC plate was scraped off and the compound was extracted with acetone. The extract was evaporated and the residue was dissolved in methanol to make a stock solution. Total radioactivity of [1-^{3}H]1-(4-chlorophenyl)-2,2,2-trichloroethanol recovered in the methanolic solution was 101.5 mCi(3.75 GBq).

Condensation of [1-^{3}H]1-(4-chlorophenyl)-2,2,2-trichloroethanol (^{3}H-carbinol) with chlorobenzene(Scheme 1) The ^{3}H-carbinol (55.2 mCi, 2.04 GBq) (ca.50 μmol), after removal of methanol, was mixed with chlorobenzene (0.5 ml). Oleum/conc.H_2SO_4 (1/5 v/v) was added dropwise (5 drops) into the mixture while cooled with ice. Stirring was continued for one hour with ice-cooling and then at room temperature overnight. Ice was added to dilute the whole mixture and the product was extracted with ether. Preparative TLC(see Materials and Methods for the condition) gave 34.7 mCi (1.28 GBq) of [methine-^{3}H]DDT with slight contamination of [^{3}H]o,p'-DDT. Specific activity was determined as the ratio of the radioactivity of a collected HPLC fraction and the amount determined based on the UV-trace of HPLC. It was 0.77 Ci/mmol (28.49 GBq/mmol). A part of the product was further purified by HPLC. The chromatograms of the purified [methine-^{3}H]DDT are shown in Fig.1. Radiochemical purity was over 99.9%.

Reduction of phenyl trichloromethyl ketone with sodium boro[^{3}H]hydride (Scheme 1) Reduction of phenyl trichloromethyl ketone was conducted similarly as above. From sodium boro[^{3}H]hydride (ca.180 mCi, ca.6.7 GBq)(5-10 Ci/mmol, 185-370 GBq/mmol)(36-18 μmol) mixed with cold $NaBH_4$ (380 μg)(10 μmol) in 0.01 N NaOH and phenyl trichloromethyl ketone (35 mg) in isopropyl alcohol, [1-^{3}H]1-phenyl-2,2,2-trichloroethanol (180 mCi, 6.7 GBq) of specific activity ca.1.62 Ci/mmol(60.3 GBq/mmol) was obtained.

Condensation of [1-^{3}H]1-phenyl-2,2,2-trichloroethanol with benzene gave [1-^{3}H]1,1-diphenyl-2,2,2-trichloroethane (108 mCi, 4 GBq)(radiochemical yield, 60%) of ca.1.6 Ci/mmol(59.2 GBq/mmol) and of a very high radio-

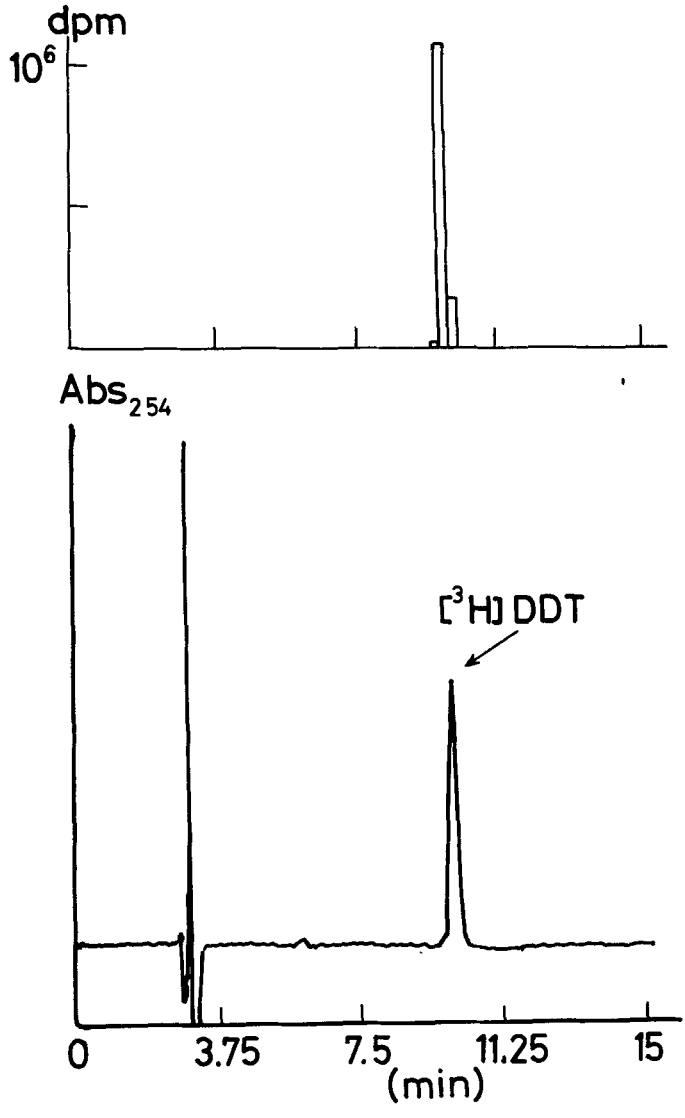


Fig.1 HPLC of [methine-^{3}H]DDT. The radiochromatogram above was obtained by collecting 20-sec fractions of the effluent and counting each fraction with an LS-counter. The chromatogram below was obtained with a conventional UV detector. HPLC solvent was dioxane/methanol/water, 3/5/2 (v/v), and the flow rate was 1 ml/min.

chemical purity (over 99.9%) according to the collected radioactivity of the HPLC fraction compared with the injected radioactivity.

[1-^{3}H]1,1-Di-(4-nitrophenyl)-2,2,2-trichloroethane([methine-^{3}H]NO_2-DDT) (Scheme 2) [1-^{3}H]1,1-Diphenyl-2,2,2-trichloroethane(44 mCi, 1.63 GBq)(7.2 mg) in benzene solution was evaporated to dryness, and the residue was mixed with acetic anhydride(1.5 ml), fumic HNO_3 (1.5 ml) and a small amount of urea while stirring below -15°C. After one-hour stirring at 0°C, ice-water was added

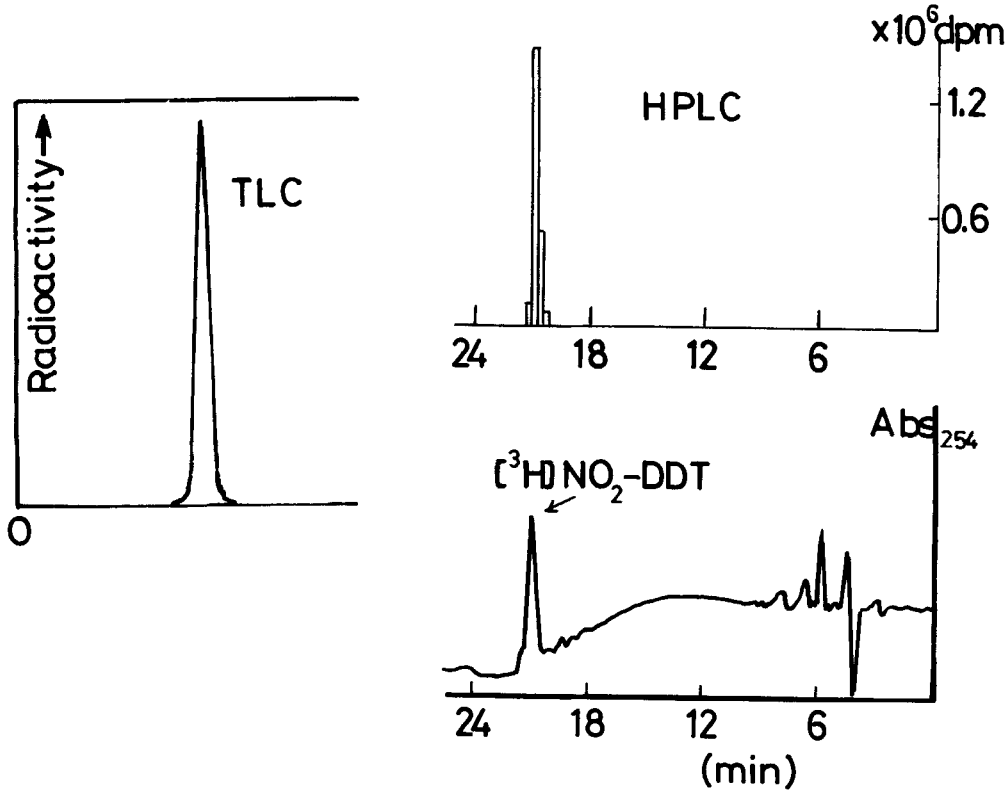


Fig.2 TLC and HPLC of [methine-^{3}H]NO_2-DDT. Radioactivity determination on TLC plates(left) was made with a position-sensitive proportional counter (Aloka chromanizer). HPLC effluents were collected and counted similarly as in Fig.1 to obtain the radiochromatogram (above right) and were also conventionally monitored with a UV-detector (below right). HPLC conditions were the same as in Fig.1.

to dilute the reaction mixture. Products were extracted with benzene and the benzene solution was evaporated after drying over Na_2SO_4. Repeated preparative TLC produced a pure sample of the title compound. TLC and HPLC

of the pure sample are shown in Fig.2. The specific activity was 1.63 Ci/mmol(60.3 GBq/mmol).

ENZYME-CATALYZED DEHYDROCHLORINATION

Enzyme Prepared as already reported (3), and air-mailed frozen in a phosphate buffer from Wellington, New Zealand to Kyoto. Stored at -70 ℃ before use.

Methods

[1] Measurement of tritium isotope effect: At 37°C, [^{3}H]DDT (or [^{3}H]-NO_2-DDT) (0.7-1.72 nmol in 20 μl DMSO) and 50 mM glutathione (GSH) (40 μl: 2 μmol) were dissolved in 1.92 ml of 0.1 M sodium phosphate buffer (pH 7.4), and an enzyme solution (20 μl: 2-3 μg protein) was added to start the reaction. After a specified period (e.g. 15 min for DDT and 4 min for NO_2-DDT), the organic metabolite [1,1-di-(4-chlorophenyl)-2,2-dichloroethylene(DDE) or its di-(4-nitrophenyl) analog (NO_2-DDE)] and the substrate were extracted with hexane/ethyl acetate 4/1 (v/v) (4 ml x 3). The organic layer was used to gas-chromatographically quantitate the DDE-type metabolite. Radioactivities in the organic layer ([^{3}H]substrate) and in the aqueous layer ([^{3}H]HCl and [^{3}H]H_2O) were determined by counting an aliquot from each layer with a liquid scintillation counter. Quenching correction was made by an external standard-channel ratio method.

As control runs, the reactions without enzyme solution (instead of enzyme solution, 20 μl of buffer solution added) were conducted. The radioactivity in the aqueous solution of a control run after a similar procedure as above was subtracted (less than 9% of the aqueous solution of the sample in case of DDT, and 5-10% in case of NO_2-DDT) from that in the aqueous layer of each sample run.

The tritium isotope effect on the $\underline{V}_{max}/\underline{K}_m$ [$=^T(\underline{V}/\underline{K})$] of the reaction was determined from the equation(1)

$$^T(\underline{V}/\underline{K}) = \ln[1-(P_H/S_H)] \;/\; \ln[1-(P_T/S_T)].$$

In this equation, P_H is the amount of DDE (or NO_2-DDE) formed from untri-

tiated DDT (or NO_2-DDT). P_T is that of DDE (or NO_2-DDE) formed from [^{3}H]DDT (or [^{3}H]NO_2-DDT). P_H+P_T is the amount of total metabolite and was determined with a gas chromatograph. P_T was calculated from the radioactivity of the aqueous layer that corresponded to [^{3}H]HCl formed. S_H and S_T are the amounts of untritiated DDT (or NO_2-DDT) and of [^{3}H]DDT (or [^{3}H]NO_2-DDT), respectively and were determined similarly to P_H and P_T. Duplicate or triplicate runs were carried out for each substrate.

[2] Measurement of deuterium isotope effects by a competitive method: The reactions were conducted similarly to the above tritium experiments. Substrates are the mixture of [methine-^{2}H]DDT and [phenyl-U-^{14}C]DDT at the molar ratio of 3:1. The deuterium isotope effect $^D(V/K)$ on the reaction was determined from the equation

$$^D(V/K) = \ln[1-(P_H/S_H)] \;/\; \ln[1-(P_D/S_D)]$$

in which S_H, S_D, P_H and P_D are the amount of the undeuteriated substrate, that of deuteriated substrate, that of the product formed from the undeuteriated substrate and from the deuteriated substrate, respectively. S_H was determined from the C-14 radioactivity of DDT, S_D by the added amount, P_H from the C-14 radioactivity of DDE recovered from HPLC after the reaction, and P_D from the difference of total DDE (with GC) and P_H.

Results and Discussion The tritium and deuterium isotope effect values on the enzyme-catalyzed dehydrochlorination of DDT and NO_2-DDT are shown in Table 1. For DDT, $^D(V/K)$ was 4.19±0.34. Based on the following Northrop's equation

$$[^D(V/K) - 1]/[^T(V/K) - 1] = (^Dk - 1)/(^Dk^{1.442} - 1)$$

we can calculate the intrinsic deuterium isotope effect ^Dk on DDT-dehydrochlorination as 13.2. (For the NO_2-DDT analog, We conducted several separate runs of kinetic studies using methine-deuteriated and undeuteriated substrate in a non-competitive mode and obtained the values between 1.58 and 2.32 for observed deuterium isotope effect values on V/K. As we described in a previous paper (3), these isotope effects on V/K are obtained for another preparation of the enzyme that was somewhat differently purified and also are not very reliable

because of the biphasic nature of 1/v vs. 1/s plot of the reaction. Since we have not yet performed a competitive type deuterium isotope effect study on the NO_2-analog, the calculated intrinsic deuterium isotope effect values for this substrate seem not reliable.)

The calculated value for the intrinsic isotope effect on DDT dehydrochlorination was as large as the theoretical maximum for deuterium isotope effect values, and strongly suggests a highly symmetrical transition state structure in the enzyme-catalyzed dehydrochlorination.

Table 1 Isotope effect on $\underline{V}/\underline{K}$ of the enzyme-catalyzed dehydrochlorination of DDT and its NO_2-analog[a)]

	$^3H(T)$	$^2H(D)$	D_k[b)]
DDT	11.51 ± 0.52	4.19 ± 0.34[c)]	13.2
NO_2-DDT	11.3 ± 1.2	-[d)]	-

a) Average values of two or three determinations with standard deviations are shown.
b) Calculated based on the Northrop's equation (see text).
c) In the experiments of the mixture at the molar ratio of $^2H/^1H = 3$.
d) A non-competitive method that is based on the comparison of the slope of double reciprocal plots 1/v vs. 1/s gave a series of values between 1.58 and 2.32.

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