DDT-Dehydrochlorinase, an Enzyme Found In DDT-Resistant Flies

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An enzyme extractable from various strains of DDT-resistant houseflies catalyzes the dehydrochlorination of DDT to form a nontoxic product [1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene]. The enzyme requires activation by glutathione. It is irreversibly inhibited at pH 3.5 or lower and has maximum activity at about pH 7.4. Temperatures much above 37° C.—e.g., 43° C.—reduce the activity of the enzyme to a point where it cannot be measured accurately, and at 27° C. its activity is about half of that at 37° C. The initial rate and time of continued reaction are better sustained under an atmosphere of nitrogen than air. The enzyme has a high degree of specificity and appears to attack only those analogs of DDT which may be sterically similar to DDT. The rate of dehydrochlorination by treatment with alkali does not correlate with the rate of enzymatic dehydrochlorination of analogs of DDT. Present methods of extraction and evaluation have demonstrated the existence of the enzyme in all strains of resistant flies available and have not indicated its presence in susceptible strains of flies.

STERNBURG AND KEARNS (*10*) and Perry and Hoskins (*7*) found that certain strains of houseflies resistant to DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] had the ability to convert a considerable quantity of the DDT absorbed from topical applications to a product which responded to the Schechter-Haller (*8*) test in a manner identical to a nontoxic degradation product, 1,1-dichloro - 2,2 - bis(*p* - chlorophenyl)-ethylene (DDE). Sternburg *et al.* (*11*) demonstrated that a certain strain of DDT-resistant flies possessed an enzyme which apparently catalyzed the dehydrochlorination of DDT to DDE and possibly to other compounds not susceptible to the Schechter-Haller test. It has since been found that the only product of enzymatic attack on DDT is DDE. This became apparent when a change was made in the procedure of extraction and analysis of the reaction mixture.

Recent work has shown that all DDT-resistant strains of houseflies available to the authors possess this enzyme, whereas it has not been found in measurable quantities in any susceptible strains. It would appear that this enzyme must be one of the factors causing resistance to DDT. The authors propose to call this enzyme "DDT-dehydrochlorinase." The present report deals with some of its reaction characteristics.

Experimental

Enzyme Preparation

Extract A. It was found convenient and simple to prepare so-called "acetone powders" of flies for storage and subsequent use as a source of DDT-dehydrochlorinase. A number of flies are anesthetized with carbon dioxide and their weight is determined. The flies are then added to the equivalent of 10 volumes of acetone, precooled to −10° C., in a refrigerated Waring Blendor, and ground for 1 minute. The resulting slurry is filtered on a cold Büchner funnel. Before the last trace of acetone passes through the filter cake, an equal volume of freshly distilled ethyl ether is added to the funnel and filtered through the cake. The filter cake is removed while still wet with ether and transferred to a vacuum desiccator, where the last trace of solvent is quickly removed under reduced pressure. The resulting powder may be retained at least one year as a source of active DDT-dehydrochlorinase, if stored in an air-tight bottle in a refrigerator.

For most of the work included in this report a partially purified solution of DDT-dehydrochlorinase was prepared from an acetone powder of *S*-strain (*3*) DDT-resistant flies. Ten grams of powder were added to 150 ml. of cold glass-distilled water. The mixture was slowly agitated by a mechanical stirrer for 3 hours at near 0° C., then centrifuged at 5000 r.p.m. in a refrigerated centrifuge for 15 minutes. The supernatant liquid, containing most of the DDT-dehydrochlorinase, was decanted from the precipitate and treated with ammonium sulfate to bring it to 30% of saturation at 0° C. The mixture was then adjusted to pH 4 with 0.5*N* hydrochloric acid. A heavy precipitate formed, which was centrifuged off and discarded. The supernatant liquid was then dialyzed in a cellophane bag for 4 hours against cold distilled water. During this time a precipitate formed which was inactive and was discarded by centrifugation. The supernatant liquid was again dialyzed against 0.075*M* sodium acetate for 12 hours in the cold. The volume of the extract was then adjusted to 150 ml. with 0.075*M* sodium acetate.

This preparation, which was used in many of the experiments reported here, contained 3.2 mg. of protein per ml. as estimated by the method of Warburg and Christian (*13*). It may also be considered to contain the equivalent in DDT-dehydrochlorinase of 17 flies per ml. The preparation is referred to as Extract A.

Extract B. DDT-dehydrochlorinase was prepared by grinding 100 grams of *S*-strain DDT-resistant flies in a Waring Blendor for 1 minute in 250 ml. of cold distilled water. The homogenate formed by this process was strained through two thicknesses of cheesecloth to remove gross particles of tissue and cuticle, and was then treated with ammonium sulfate to bring it to 35% of saturation at 0° C. The precipitate that formed was discarded by centrifugation. The supernatant liquid was then treated with additional ammonium sulfate to bring it to 55% of saturation. The resultant precipitate contained most of the DDT-dehydrochlorinase. This was separated from the supernatant liquid by centrifugation and put into solution in approximately 50 ml. of distilled water. This solution was then

Table I. Dehydrochlorination of Analogs of DDT by DDT-Dehydrochlorinase

(Reactions contained 4 mg. of substrate on 600 mg. of glass beads, 2 ml. of Extract A, 1 ml. of 0.137*M* phosphate buffer at pH 7.4, and 0.003*M* glutathione. All reactions run 2 hours at 37° C. under N_2)

Substrate	Substrate Enzymatically Dehydrochlorinated, γ	Hydrolysis Rate Constant 10^5K, Liters/Sec./Mole, 37° C. (12)
1,1,1-Trichloro-2-bis-(*p*-chlorophenyl)ethane (DDT)	534	12,515
1,1,1-Trichloro-2,2-bis-(*p*-bromophenyl)ethane	345	18,760
1,1-Dichloro-2,2-bis-(*p*-chlorophenyl)ethane (TDE)	330	4,035
1,1,1-Trichloro-2,2-bis-(*p*-tolyl)ethane	47	75.6
1,1,1-Trichloro-2,2-bis-(*p*-methoxyphenyl)ethane-(methoxychlor)	230	76.8
1,1,1-Trichloro-2,2-bis-(*p*-iodophenyl)ethane	70	19,800
1,1,1-Trichloro-2,2-bis-(phenyl)ethane	20[a]	272
1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane	0	255
1,1-Dichloro-2,2-bis-(*p*-chlorophenyl)ethylene (DDE)	0	0

[a] Amount not very significant in proportion to amount of parent product in extract subjected to analysis.

dialyzed in the cold against distilled water for about 12 hours, during which time the water was changed four times. A small precipitate gradually formed and was discarded after centrifugation. The supernatant liquid was made up to a volume of 100 ml. with distilled water.

This extract may be considered to contain the equivalent in DDT-dehydrochlorinase of 50 flies per ml. or 23.6 mg. of protein per ml. This particular extract is designated as Extract B.

For rough comparative studies of DDT-dehydrochlorinase activity in different strains of flies, homogenates were made by grinding 75 flies in 25 ml. of cold distilled water for 1 minute. The gross particles of tissue and cuticle were removed by pressing the homogenate through two thicknesses of cheesecloth. Tests made upon such preparations are reported as homogenates of certain strains of flies, and the enzyme equivalent of a certain number of flies per milliliter of reaction mixture is indicated in the results recorded.

DDT-Dehydrochlorinase Reactions All reactions were carried out in single side-arm Warburg flasks of 15-ml. capacity. The DDT was applied in acetone solution to glass beads placed in the bottom of the flask and the solvent was evaporated in an air stream. About 100 mg. of beads 15 to 60 microns in diameter were required to give the maximum rate of dehydrochlorination for 1.0 mg. of DDT. This method of substrate exposure and ratio of beads to DDT were used in all experiments unless otherwise noted.

Aliquots of enzyme extracts were diluted with distilled water to a concentration where 2 ml. of the solution would give the desired enzyme concentration when eventually made up to a volume of 3 ml. with 0.137*M* phosphate buffer containing glutathione. Thus 2 ml. of solution containing the desired concentration of enzyme was added to a flask containing DDT crystallized on glass beads, and to it was added 0.5 ml. of 0.137*M* phosphate buffer. An additional 0.5 ml. of phosphate buffer containing 0.018*M* glutathione was placed in the side arm of the reaction flask, which was then attached to a manometer and placed in a water bath regulated to the desired temperature. The contents of the flask were allowed to equilibrate with respect to temperature for 15 minutes, during which time they were constantly shaken under a stream of nitrogen. At the end of this time the glutathione solution was tipped into the reaction mixture. This was considered to be zero time for all reactions.

The reaction mixture thus contained 2 ml. of enzyme solution, 1 ml. of 0.137*M* phosphate buffer at pH 7.4, and 0.003*M* concentration of glutathione. The reactions were allowed to run for the desired period of time with constant shaking under an atmosphere of nitrogen.

Extraction and Analysis After the flask had been removed from the manometer, the neck of the flask was degreased with cleansing tissue moistened with cyclohexane. Three milliliters of sulfuric acid were added to the flask to stop the reaction and prevent emulsions from forming during the extraction process. After the flask had cooled, 8 ml. of cyclohexane was added, and the flask was stoppered and shaken vigorously for 30 minutes on a mechanical shaker. An aliquot of the cyclohexane layer was taken and diluted with cyclohexane until the expected total of DDT and DDE fell within the range of 15 to 25 γ per ml. The absorbance of these solutions was then determined at 241 and 260 mμ in silica cells with a 1-cm. light path in the Beckman DU spectrophotometer against a blank of cyclohexane. At 241 mμ there is equal absorbance by DDT and an equivalent molar concentration of DDE. Therefore the absorbance at 241 mμ is directly

Table II. Wave Lengths of Maximum Absorbance, Isosbestic Point, and Point of Reference

(Used in determination of DDT and various analogs and their corresponding dehydrochlorination products. Absorbance measured in silica cells with 1-cm. light path in Beckman DU spectrophotometer. Cyclohexane solutions contained 23.8 γ per ml. of DDT or analogous compound. Absorbance of corresponding dehydrochlorinated products measured in molar equivalents to their parent compounds)

	Max.	A	Isos.	A	Ref.	A
1,1-Dichloro-2,2-bis-(*p*-chlorophenyl)ethane	231.0	1.655	235.5	1.415	260	0.053
1-Chloro-2,2-bis-(*p*-chlorophenyl)ethylene	244.0	1.671	235.5	1.415	260	1.271
1,1,1-Trichloro-2,2-bis-(*p*-chlorophenyl)-ethane	237.0	1.303	241.0	1.156	260	0.050
1,1-Dichloro-2,2-bis-(*p*-chlorophenyl)ethylene	247.0	1.238	241.0	1.156	260	1.068
1,1,1-Trichloro-2,2-bis-(*p*-iodophenyl)ethane	248.5	1.308	251.0	1.195	275	0.104
1,1-Dichloro-2,2-bis-(*p*-iodophenyl)ethylene	257.0	1.333	251.0	1.195	275	0.856
1,1,1-Trichloro-2,2-bis-(*p*-bromophenyl)-ethane	242.0	1.264	230.0	0.940	260	0.064
1,1-Dichloro-2,2-bis-(*p*-bromophenyl)-ethylene	251.0	1.304	230.0	0.940	260	1.029
1,1,1-Trichloro-2,2-bis-(*p*-methoxyphenyl)-ethane	229.0	1.220	239.0	0.890	290	0.028
1,1-Dichloro-2,2-bis-(*p*-methoxyphenyl)-ethylene	253.5	1.388	239.0	0.890	290	0.748
1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane	237.0	1.099	...	...	251	0.040
1,1-Dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethylene[a]	251.0	0.956	...	...	237	0.839
1,1,1-Trichloro-2,2-bis-(phenyl)ethane	227.0	0.842	...	...	253	0.034
1,1-Dichloro-2,2-bis-(phenyl)ethylene	253.0	0.983	...	...	227	1.133
1,1,1-Trichloro-2,2-bis-(*p*-tolyl)ethane	235.0	0.970	...	...	260	0.063
1,1-Dichloro-2,2-bis-(*p*-tolyl)ethylene	243.0	1.141	...	...	260	0.993
1,1-Dichloro-2,2-bis-(*p*-tolyl)ethylene	...	...	...	...	235	1.139

[a] Compound not chemically pure.

Table III. Recovery of DDT and DDE after Exposure of DDT to Enzyme Extract A

(Reaction mixture contained 2 ml. of enzyme solution, 1 ml. of 0.137*M* phosphate buffer at pH 7.4, and 0.003*M* glutathione. Reaction time 1.25 hours, under N_2 at 37° C., equivalent of 11.3 *S*-strain flies or 2.2 mg. of protein per ml.)

DDT Substrate, γ	Recovered, γ			% of Substrate Accounted for
	Total[a]	DDT	DDT dehydrochlorinated	
250	254	127	127	101.6
750	730	582	148	97.3
1500	1450	1200	250	96.7
2500	2490	2080	410	99.6
4000	4000	3580	420	100.0

[a] Total DDT recovered plus DDE calculated as equivalent of DDT.

proportional to the original amount of DDT present before dehydrochlorination to DDE. At 260 mμ DDT has a very low absorbance, whereas DDE absorbs strongly (*1*, *6*). This wave length was therefore chosen for the other measurement necessary to determine the amounts of the two compounds present in the mixture. The amount of each compound present in the mixture was calculated by standard methods used for two-component systems. One part of DDE may be determined in the presence of 80 parts of DDT with an error of less than 2% by this method.

Isolation and Identification of DDE A series of cyclohexane extracts from DDT-dehydrochlorinase reactions was pooled until about 185 mg. of DDT and 30 mg. of DDE were estimated to be present in 430 ml. of cyclohexane. This solution was then treated according to the method of Sternburg and Kearns (*9*) for the separation of DDT and DDE. The nearly pure DDE obtained in this manner was recrystallized twice from methanol and dried in vacuo. Its melting point was 89° C.; that of DDE is 89° C. Analysis. Calculated as $C_{14}H_8Cl_4$ (DDE): C, 52.86; H, 2.52; Cl, 44.62. Found: C, 52.91; H, 2.51; Cl, 44.28.

DDT-Dehydrochlorinase and DDT Analogs The compounds used in these experiments (Table I) were obtained from various sources or prepared by methods reported in the literature. All compounds were analyzed and known to be pure. They were exposed to DDT-dehydrochlorinase in the same manner as DDT. All the compounds and their corresponding dehydrochlorination products were found sufficiently soluble in cyclohexane to be extracted by the usual method.

The ultraviolet absorption spectra of the various analogs of DDT were similar to that of DDT, but with slightly different $\lambda_{max.}$ The corresponding dehydrochlorinated compounds were similar to DDE, but with slightly different $\lambda_{max.}$ Therefore, it was possible to determine these compounds in a manner similar to that described for DDT and DDE but differing in the wave lengths at which readings were made (Table II).

In the case of 1,1,1-trichloro-2,2-bis-(phenyl)ethane and 1,1,1-trichloro-2,2-bis-(*p*-tolyl)ethane and their corresponding dehydrochlorinated products, absorbance was measured at the point of maximum absorbance of the parent compound and at a wave length of minimum interference by this compound with its dehydrochlorinated product (Table II), and these values were used to determine the amount of enzymatic dehydrochlorination.

Interference

The accuracy of this method of analysis depends upon the elimination of compounds causing interference in the ultraviolet region where readings are made. In most of the work on DDT-dehydrochlorinase for which this method was developed, the enzyme had been obtained from acetone-ether powders of houseflies. In the preparation of these powders nearly all compounds soluble in cyclohexane causing interference in the ultraviolet were removed. Homogenates from live flies not subjected to acetone powdering have somewhat more absorption than preparations made from acetone powders, but as it is possible to subject an identical amount of the enzyme preparation used in any one experiment to the same extraction procedure, a reliable blank may be obtained to correct for interference.

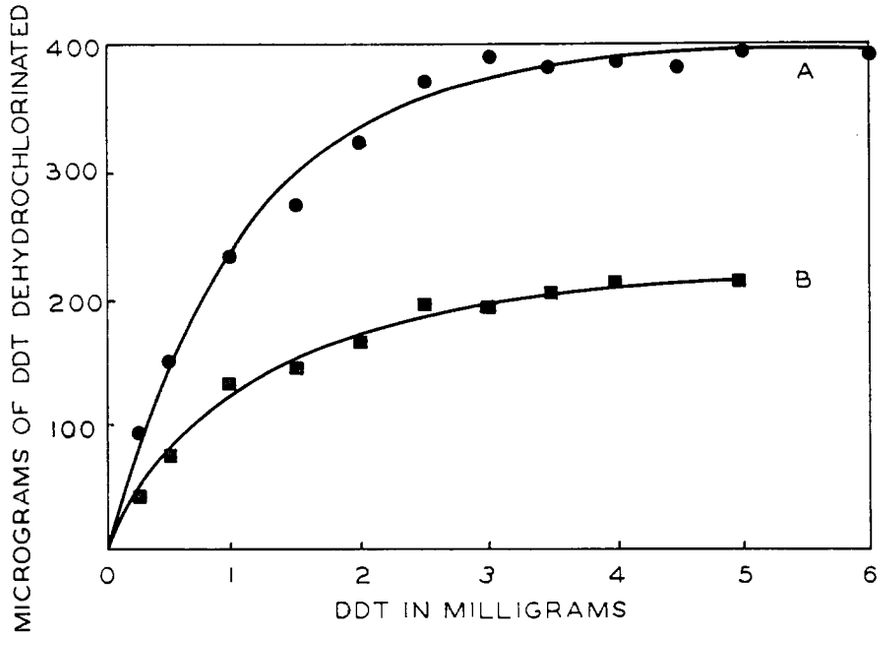
Glutathione Determinations. The percentage of the original quantity of glutathione remaining in reaction mixtures after various times of incubation was determined by the method described by Bray *et al.* (*2*) for use under very similar conditions. These determinations could not be made on the reaction mixtures used to determine the dehydrochlorination of DDT, but were made on duplicate mixtures.

Results and Discussion

The only product obtained from the attack of DDT-dehydrochlorinase on DDT is DDE. This is indicated by the fact that reactions consistently yield near theoretical quantities of DDT if the product is assumed to be DDE (Table III). Further evidence is found in the fact that the enzyme does not attack DDE under conditions most favorable for the dehydrochlorination of DDT (Table I). Proof that DDE is a product

Figure 1. Effect of substrate concentration on DDT-dehydrochlorinase

Reaction mixture contained 2 ml. of Extract A (equivalent of 11.2 *S*-strain flies or 2.2 mg. of protein per ml.), 1 ml. of 0.137M phosphate buffer to hold pH 7.4, 0.003M glutathione, and DDT on 600 mg. of glass beads. Reactions run under N_2 for 75 minutes, *A* at 37° C. and *B* at 27° C.



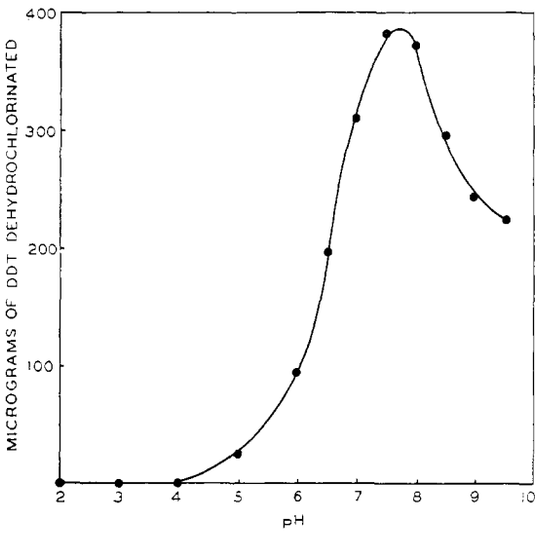


Figure 2. Effect of pH on DDT-dehydrochlorinase

Reaction mixture contained 2 ml. of Extract A (equivalent of 11.3 S-strain flies or 2.2 mg. of protein per ml.), 1 ml. of phosphate buffer, 0.003*M* glutathione, 4 mg. of DDT on 600 mg. of glass beads. Reactions run for 75 minutes under N_2 at 37° C.

of the reaction was established by the isolation and identification of the compound from a series of pooled enzyme-catalyzed reactions.

The reaction by which DDT is enzymatically dehydrochlorinated in vitro apparently occurs at the interface of solid DDT and the enzyme solution rather than being involved with the minute amount of DDT which might be soluble in the aqueous phase. The smallest amount of DDT used in this study (66 mg. per liter) is far in excess of that required to saturate the aqueous solution (0.1 mg. per liter) (*5*), yet considerably less than that required to approach the maximum reaction velocity (1.33 grams per liter) (Figure 1). This being so, it should follow that the reaction characteristics of DDT-dehydrochlorinase would vary not only in respect to the amount of substrate available, but also in accordance with the surface area of the substrate exposed to attack.

If the substrate is exposed to the enzyme as a crystalline layer on the bottom of a reaction flask by simply allowing acetone to evaporate from a solution of DDT, only a small amount of DDT is dehydrochlorinated, even though large amounts of substrate are used (Table IV). A greater amount of DDT is dehydrochlorinated when it is allowed to precipitate as a semicolloidal suspension in the enzyme extract from an ethyl alcohol solution. The amount of DDT which may be added in this manner is limited, because DDT is only moderately soluble in ethyl alcohol (2% at 25° C.) and the enzyme will not withstand a concentration of ethyl alcohol greater than 0.02% at 37° C. Acetone and other water-miscible solvents are even more destructive under such conditions and thus could not be used.

Numerous attempts have been made to prepare a satisfactory homogeneous reaction mixture, without success. What would appear to be a homogeneous mixture may be attained by dissolving DDT in Tween 80 (water-miscible surface active agent and DDT solvent manufactured by the Atlas Powder Co., Wilmington, Del.) and then adding this to the enzyme solution. Dehydrochlorination of DDT will not occur in such mixtures, possibly because of inhibition of the enzyme by Tween 80. DDT is attacked by the enzyme when added to the reaction mixture as a finely ground powder. Considerable variations occur from such preparation and a comparatively slow reaction rate results.

Any attempt to study the kinetics of DDT-dehydrochlorinase would require that variations due to the physical state of the substrate be reduced to a minimum. The nearest approach to a satisfactory solution of this problem which the authors have been able to devise consists of exposing DDT recrystallized from acetone solution on glass beads 15 to 60 microns in diameter (pavement marking beads No. 119, Minnesota Mining and Manufacturing Co., Minneapolis, Minn.). Under these conditions both the quantity of substrate and its surface area are factors controlling the rate of reaction. This complication may be simplified by using enough beads (approximately 100 mg. of beads per mg. of DDT) to provide an excess of surface area for the substrate, and thus shift the control to the concentration of substrate as shown in Table IV. By this means variations in reaction rate for any given enzyme preparation are reduced to ±10%, and it is possible to measure quantitatively other effects upon the enzymatic dehydrochlorination of DDT.

Like most enzymes, DDT-dehydrochlorinase has a narrow pH range through which it exerts its maximum effect, as shown in Figure 2. The maximum activity is attained at about pH 7.4. A marked loss in activity occurs at pH 8.5 and 6.5. The enzyme may be exposed to pH 4 without permanently destroying its activity, but, at pH 3.5 or lower, the enzyme is irreversibly inhibited.

The rate at which DDT may be dehydrochlorinated may vary with different sources of enzyme and methods used in preparing the enzyme for use. The particular preparation used in most of the experiments herein reported (*A*, Figure 3) appears to sustain a constant rate of reaction for about the first 90 minutes, after which the reaction changes and the velocity diminishes. Other enzyme preparations may sustain a linear reaction rate for as long as 3 hours (*C*, Figure 3) or for only 60 minutes (*D*, Figure 3). The cause for this variability is not known. It is, however, important to determine this characteristic for any given preparation if a quantitative or comparative study is contemplated. Comparative DDT-dehydrochlorinase activity should be measured at time intervals during which the reaction mixtures sustain a constant rate. Beyond this period the order of reaction becomes very complicated and unpredictable.

Enzymatic dehydrochlorination of DDT is proportional to the amount of enzyme present for a wide variety of time intervals, providing glutathione and substrate are present in sufficient amounts to sustain some order of reaction during this time, as shown in Figure 4. This characteristic holds true for various enzyme preparations. It should, therefore, be possible to evaluate different steps or attempts to purify and concentrate DDT-dehydrochlorinase on this basis.

The effect of substrate concentration on the quantity of DDT dehydrochlorinated is shown in Figure 1. The relationship of substrate concentration and

Table IV. Effect of Physical State of DDT on Amount of DDT Enzymatically Dehydrochlorinated

(Reactions run at 37° C. for 2 hours, under N_2. Reaction mixture contains 2 ml. of enzyme Extract A, 1 ml. of phosphate buffer at pH 7.4, and 0.003*M* glutathione)

Physical State of Substrate	Substrate DDT, Mg.	DDE Produced, γ
Finely ground powder suspended in enzyme solution	1.0	27
	2.0	25[a]
	3.0	40
	4.0	40[a]
Precipitated in enzyme solution from ethyl alcohol solution[b]	0.1	25
	0.2	65
	0.4	105
	0.8	210
Recrystallized from acetone on bottom of reaction flask	1.0	25
	2.0	20[a]
	3.0	35[a]
	4.0	40[a]
On 200 mg. of glass beads[c]	1.0	145
	2.0	195
	3.0	215
	4.0	235
On 600 mg. of glass beads	1.0	225
	2.0	300
	3.0	305
	4.0	320
Solution in Tween 80[d]		
0.5 ml.	4.0	0
0.1 ml.	4.0	0

[a] Amount of DDE too little in proportion to DDT to have much significance.

[b] Amount of DDT limited by low solubility of ethyl alcohol (2%) and denaturing effects of solvent on enzyme.

[c] Glass beads 15- to 60-μ diameter, No. 119 pavement marking beads, Minnesota Mining & Mfg. Co., Minneapolis, Minn.

[d] Tween 80 DDT solvent and water-soluble surface active agent of Atlas Powder Co., Wilmington, Del.

enzyme activity graphically appears as a rectangular hyperbola not unlike that which is characteristic of other enzyme-substrate relationships. DDT-dehydrochlorinase would appear to follow the so-called Michaelis-Menten kinetics, and from these data it is possible to calculate a Michaelis constant and determine the maximum velocity of the reaction. The significance of such values would have to be restricted to the particular method of substrate exposure used in the experiments, and they may not have the significance usually attributed to these constants, where the attack of the substrate by the enzyme occurs in a homogeneous reaction mixture. It is believed that a study of enzyme substrate relationships in greater detail should await development of purer enzyme preparations and methods for following the reaction rates at closer intervals.

There is no evidence that excessive quantities of DDT may inhibit DDT-dehydrochlorinase. The most concentrated enzyme extracts used in these experiments attained their maximum velocity when exposed to about 1 mg. of DDT per ml. of reaction mixture. Twice this amount of DDT had no effect upon the rate of the reaction (Figure 1).

The initial velocity of the reaction is approximately the same, whether the reaction is carried out under air or nitrogen. However, the reaction continues for a much longer time under an atmosphere of nitrogen (Table V) The glutathione content of the reaction mixtures remains at what is believed to be a sufficient level (0.003 to 0.001M) to sustain the initial reaction velocity in either case. Failure of these reactions to continue undiminished is not due to lack of substrate, because sufficient substrate remains after the longest reaction period and where the greatest amount of DDE is produced. As DDE does not inhibit the enzyme and other factors remain favorable, it is believed that enzyme deterioration is the cause of the observed decrease in reaction rate. If so, the enzyme deteriorates much more rapidly under air than under nitrogen.

The role of glutathione in the reactions of DDT-dehydrochlorinase is not understood. It cannot be replaced by other sulfhydryl compounds such as cysteine or BAL 2,3-dimercapto-1-propanol (*11*). Glutathione is essential to activate extracts of DDT-dehydrochlorinase prepared by the methods described herein. Occasionally homogenates of DDT-resistant flies will dehydrochlorinate DDT without the addition of glutathione. This indicates that in the usual process of extracting the enzyme, the glutathione or its equivalent which is contained by the fly is inactivated or otherwise lost. Thus the amount of glutathione required to activate an enzyme preparation may be presumed to vary, depending upon the amount of glutathione retained in the preparation from the flies used as sources of enzyme. It has been shown (*11*) that glutathione concentrations varying from 0.001 to 0.006M are equally effective in the activation of the enzyme. Reactions run as long as 5 hours under either air or nitrogen do not fall below 0.001M glutathione when the original concentration is 0.003M, as shown in Table V. Reactions run for as long as 5 hours under an atmosphere of nitrogen retain approximately their original concentration of glutathione. As the compound is not measurably used up in the dehydrochlorination of DDT and in all cases was used in excess of the minimum, it need not be considered as a variable in the interpretation of the effect of other factors upon DDT-dehydrochlorinase.

Temperature has a pronounced effect upon the activity of DDT-dehydrochlorinase. The enzyme has a Q_{10} (27° to 37° C.) of approximately 2 for various concentrations of substrate ranging from 66 γ to 2 mg. per ml. (Figure 1). The activity of the enzyme is diminished at a temperature of 43° C. to a point where it cannot be measured accurately.

Various strains of DDT-resistant and susceptible houseflies collected from different laboratories in this country were tested for the presence of DDT-dehydrochlorinase (Table VI). The results do not permit precise quantitative treatment, because the amount of substrate used (66 γ per ml. of reaction mixture) is far from sufficient to attain the maximum reaction velocity from flies which possess relatively large quantities of DDT-dehydrochlorinase. The small quantity of substrate was used

Table V. Effects of Air and Nitrogen on Rate of Enzymatic Dehydrochlorination of DDT

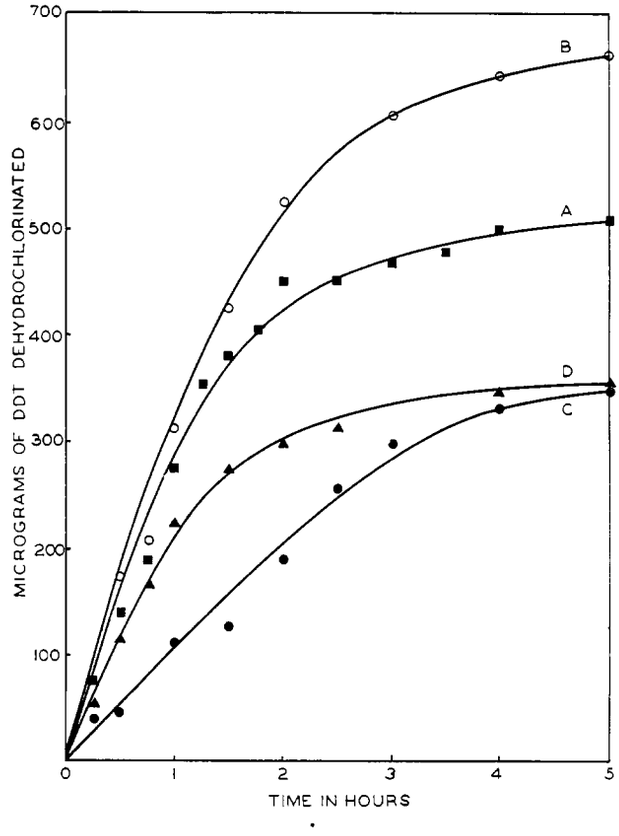
(Reaction mixtures contained 2 ml. of enzyme preparation, 1 ml. of 0.137 M phosphate buffer at pH 7.4, 4 mg. of DDT on 600 mg. of glass beads, and 0.003M glutathione. Reactions run at 37° C. Extract B, equivalent of 4 *S*-strain flies or 3.2 mg. of protein per ml.)

	DDT Dehydrochlorinated, γ		% of Original Concn. of Glutathione	
Reaction Time, Minutes	Under air	Under N_2	Air	N_2
30	50	60	90	93
60	80	90	90	96
90	100	110	84	98
120	100	135	77	92
150	100	155	63	98
300	110	235	62	98

Figure 3. Reaction rate of DDT-dehydrochlorinase on DDT

Reaction mixtures contained 4 mg. of DDT on 600 mg. of glass beads, 1 ml. of 0.137M phosphate buffer at pH 7.4, 2 ml. of enzyme solution, and 0.003M glutathione. All reactions run at 37° C. under N_2

A. 2 ml. of Extract A or equivalent of 11.3 S-strain flies and 2.2 mg. of protein per ml. of total reaction mixture
B. 2 ml. of Extract B, after 1 to 1 dilution with distilled water; equivalent of 16.6 S-strain flies or 12.7 mg. of protein per ml. of total reaction mixture
C. 2 ml. of homogenate of S-strain of flies or equivalent of 2 flies per ml. of final reaction mixture
D. 2 ml. of extract prepared from lindane strain of flies by procedure used in preparation of Extract B (lindane strain, obtained from U. S. Public Health Service, has a high level of resistance to DDT, lindane, and other chlorinated hydrocarbon insecticides)



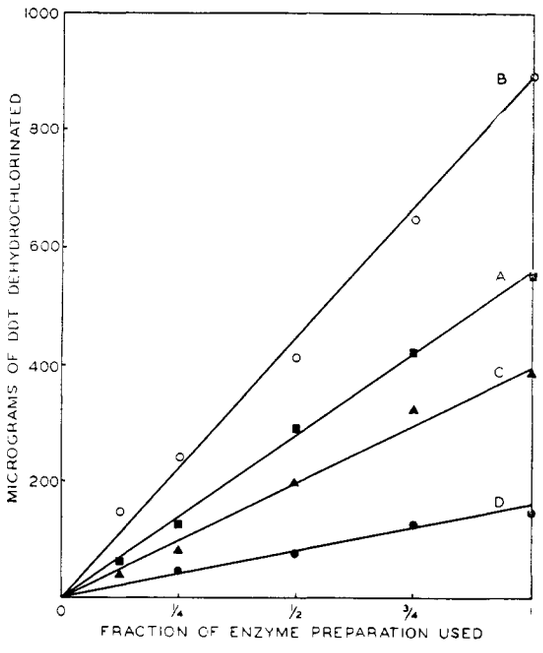


Figure 4. Effect of enzyme concentration on dehydrochlorination of DDT

Reaction mixtures contained 4 mg. of DDT on 600 mg. of glass beads, 1 ml. of 0.137*M* phosphate buffer at pH 7.4, 0.003*M* glutathione, and 2 ml. of enzyme solution or fraction therefrom

- A. Extract A, reaction period 2 hours. Reaction mixture at maximum enzyme concentration is equivalent of 11.3 S-strain and 2.2 mg. of protein per ml.
- B. Extract B, reaction period 5 hours. Reaction mixture at maximum enzyme concentration is equivalent of 20.7 S-strain flies per ml. or 15.9 mg. of protein per ml.
- C. Reaction period 75 minutes, otherwise same as A
- D. Extract B, reaction period 5 hours. Reaction mixture at maximum enzyme concentration is equivalent to 4 flies per ml. or 3.2 mg. of protein per ml.

because it was anticipated that some strains would prove to have a low concentration of enzyme and it would not be possible to measure accurately the amount of DDE produced if it were present in mixture with a large quantity of DDT. It is significant that all strains of flies which have resistance to DDT have a measurable amount of DDT-dehydrochlorinase. Those strains of flies which are susceptible to DDT do not by the same procedure demonstrate the possession of DDT-dehydrochlorinase. A strain of flies with specific resistance to dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,1,4,4a,5,6,7,8,8a-octahydro-1,4-endo, exo-5,8-dimethanonaphthalene) does not possess a significant quantity of DDT-dehydrochlorinase. The degree of resistance of flies to DDT would appear to correlate with the amount of DDT dehydrochlorinated by their homogenates. Correlation may be significant for strains displaying a small to moderate amount of activity, but for highly resistant strains lack of substrate may be a limiting factor and true differences would not be reflected in the results.

A series of analogs of DDT was subjected to the action of DDT-dehydrochlorinase to determine what structural features of the DDT molecule are related to the ability of the enzyme to catalyze dehydrochlorination (Table I). The results show that the enzyme is somewhat specific with respect to the substrate. The analogs most readily attacked were found to be those which are most toxic to flies. It would seem that the enzyme may be stereospecific in that the *o,p*-isomer of DDT is not attacked nor is 1,1,1-trichloro-2,2-bis-(phenyl)ethane, in which the ring substituents are absent. This suggests the possibility that *p*-substituents in the rings may be necessary and of some steric significance for the attachment of enzyme to substrate.

The relative ease of dehydrochlorination of DDT and analogous compounds under treatment with alkali does not correlate with the rate of dehydrochlorination induced by DDT-dehydrochlorinase. The *p,p'*-diiodo analog (Table I) is most readily dehydrochlorinated by alkali, yet it proved to be one of the most resistant to DDT-dehydrochlorinase. Therefore, data pertaining to the relative ease with which these compounds dehydrochlorinate with alkali should not be used as a basis for speculating upon their fate when applied to insects (*4*) or subjected to attack by an enzyme.

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Literature Cited

(1) Andrews, H. L., White, W. C., Gamow, L. R., and Peterson, D. C., *U. S. Public Health Repts.*, **61,** 450 (1946).
(2) Bray, H. G., Thorpe, W. V., and Vallance, D. K., *Biochem. J.*, **51,** 193 (1952).
(3) Bruce, W. N., and Decker, G. C., *Soap*, **26,** 122 (1950).
(4) Busvine, J. R., *Nature*, **168,** 193 (1951).
(5) Guavadan, P., and Poussel, H., *Compt. rend.*, **224,** 683 (1947).
(6) Herriott, R. M., *Science*, **104,** 228 (1946).
(7) Perry, A. S., and Hoskins, W. M., *Ibid.*, **111,** 600 (1950).
(8) Schechter, M. S., Soloway, D. B., Hayes, R. A., and Haller, H. L., *Ind. Eng. Chem., Anal. Ed.*, **17,** 704 (1945).
(9) Sternburg, J., and Kearns, C. W., *J. Econ. Entomol.*, **45,** 505 (1952).
(10) Sternburg, J., Kearns, C. W., and Bruce, W. N., *Ibid.*, **43,** 214 (1950).
(11) Sternburg, J., Vinson, E., and Kearns, C. W., *Ibid.*, **46,** 513 (1953).
(12) Von Oettingen, W. F., and Sharpless, N. E., *J. Pharmacol. Exptl. Therap.*, **88,** 400 (1946).
(13) Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1941).

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Table VI. DDT-Dehydrochlorinase Activity in Homogenates of Different Strains of DDT-Resistant and Susceptible Flies

(Reaction mixtures contain 2 ml. of homogenate equivalent to 6 flies per ml., 0.003*M* glutathione, 200 γ of DDT in 50 γ of ethyl alcohol, and 1 ml. of 0.137*M* phosphate buffer to hold pH 7.4. All reactions run under N_2 at 37° C.)

Strain	Est. LD_{50},[a] γ DDT/Fly	Reaction Time, Minutes					
		30	60	90	120	240	480
		DDT Dehydrochlorinated, γ					
C.S.M.A.[b]	0.25	0	0	0	0	0	0
Lab. 1[c]	0.3	0	0	0	0	0	0
NAIDM[d]	0.25	0	0	0	0	0	0
Pollard[d]	1.85[e]	0	0	0	0	12	15
Bellflower[d]	1.50[e]	0	0	0	0	0	12
Orlando[f]	2.0[e]	0	0	0	12	18	20
Lindane[g]	75.0	18	27	34	45	63	78
DDT-45[g]	55.0	16	24	32	51	60	64
Super Pollard[d]	70.0	16	20	..	32	58	77
DMC-5X[g]	50.0	12	25	40	45	46	52
Roberds[g]	25.0	5	10	15	20	24	35
C.W.S.[h]	40.0	0	5	7	10	15	25
Dieldrin[g]	1.5	0	0	0	0	8	12
CSMA[d]	0.25	0	0	0	0	0	0
S-strain[c]	70.0	16	23	37	51	62	74

[a] LD_{50} for strains of flies determined by topical applications of acetone solutions of DDT to flies.
[b] Obtained from Chemical Specialties Manufacturers Association.
[c] Obtained from W. N. Bruce, Illinois State Natural History Survey, Urbana, Ill.
[d] Obtained from R. B. March, Citrus Experiment Station, University of California, Riverside, Calif.
[e] Strains of flies formerly high in resistance, but allowed to revert back to near normal susceptibility by supplier.
[f] Obtained from Harry Dietz, Pest Control Division, E. I. du Pont de Nemours & Co., Wilmington, Del.
[g] Obtained from Richard Fay, U. S. Public Health Service, Savannah, Ga.
[h] Obtained from Leigh Chadwick, Chemical Warfare Center, Edgewood, Md.