parathion were observed at the highest temperature. For several days following decontamination, more perspiration was noted on the exposed area than elsewhere on the body.

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INSECT METABOLISM OF INSECTICIDES

The Enzymatic in vitro Degradation of DDT by Susceptible and DDT-Resistant Body Lice

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Homogenates and acetone powders of both susceptible and DDT-resistant body lice catalyze the degradation of DDT in vitro. Reduced glutathione, cysteine, ascorbic acid, thioglycollic acid, and coenzyme A may be used as cofactors for activation of the enzyme system. Enzyme preparations when incubated with DDT under optimum conditions yield at least three metabolites. On the basis of their neutral or acidic character, ultraviolet and infrared absorption spectra, colorimetric analysis, and paper chromatography, the metabolites have been identified as 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (DDE); 4,4-dichlorobenzophenone (DBP); and bis-(*p*-chlorophenyl)acetic acid (DDA). This is the first demonstration of DDA as a product of DDT metabolism in an insect.

DETOXICATION of DDT to innocuous derivatives has been implicated in the resistance of certain insect species to this insecticide, while in other species this correlation has not been conclusive. Perhaps the most challenging and fruitful investigations in elucidating the mechanism of DDT resistance have been those dealing with the metabolism of DDT by the housefly *Musca domestica* L. (*18*).

The demonstration of the enzymatic dehydrochlorination of DDT by DDT-resistant houseflies (*25*) and the subsequent isolation of the enzyme DDT-dehydrochlorinase (*24*) and its purification (*11*, *12*, *15*), have greatly strengthened the hypothesis that dehydrochlorination of DDT is an important factor in the over-all protective mechanism against the lethal action of this insecticide toward houseflies.

An enzyme system catalyzing the dehydrochlorination of DDT, DDD, and methoxychlor to their corresponding ethylene derivatives has been isolated from Mexican bean beetle larvae and pupae (*6*, *26*, *27*). Also, an oxidizing enzyme system capable of converting DDT to 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethanol has been demonstrated in the microsomal fractions of tissue homogenates of German and American roaches, the housefly, and the house mosquito (*1*). However, attempts to demonstrate the in vitro dehydrochlorination of DDT by DDT-resistant mosquitoes (*5*, *17*) and by the vinegar fly *Drosophila melanogaster* (*28*) have been unsuccessful.

In a previous communication, Perry and Buckner (*19*) reported on the in vivo metabolism of DDT by susceptible and DDT-resistant body lice *Pediculus humanus humanus* L. By incorporating C^{14}-DDT into citrated human blood and feeding it to adult lice through freshly dissected chickskin membranes, these authors showed that the DDT-resistant lice (Korean strain) but not the susceptible lice (Orlando strain) metabolized DDT to a nontoxic acidic conjugate. On the other hand, crude enzyme preparations of both strains of lice metabolized DDT in vitro at an approximately equal rate.

This report covers the methods used and the results obtained in isolating the enzyme system and in identifying degradation products resulting from the in vitro metabolism of DDT by susceptible and DDT-resistant body lice.

Materials and Methods

Enzyme Preparations. Homogenates and acetone powders were used for most of this work. Homogenates were prepared by macerating 3000 to 5000 adult lice of known weight in a mortar containing 3 ml. of a mixture of 0.1*M* NaCl and 0.1*M* $NaHCO_3$. The slurry was further homogenized in a blender at 14,000 r.p.m. for 2 minutes, after which it was strained through two gauze pads to remove gross particles and centrifuged at 14,000 × G in a refrigerated centrifuge. The precipitate contained very little activity and was discarded. The supernatant was adjusted with additional saline-bicarbonate to 10% concentration (1 gram of lice per 10 ml. of supernatant).

Acetone powders were prepared by a procedure similar to that described by Binkley (*3*) and Binkley *et al.* (*4*) for renal peptidases. Several thousand adult lice of known weight were triturated with acetone. After steeping for several hours at room temperature, the supernatant acetone was decanted, fresh acetone was added, and the mixture was boiled gently in a hot water bath at 70° C. for 20 minutes.

The supernatant acetone was removed, and the boiling with fresh acetone was repeated twice. Finally, the mixture was filtered while hot and the precipitate dried in vacuo. The resulting cake was pulverized and stored in an airtight container at −10° C. until used. For enzyme assay, 1 gram of powder was

triturated with 20 ml. of saline-bicarbonate. This preparation was flash-digested for 8 hours at 50° C. with 10 mg. of pancreatin and 10 mg. of protease, after which it was centrifuged at 14,000 × G for 15 minutes. The supernatant solution containing 90% of the enzyme activity was refrigerated until use. Acetone powders and homogenates of the type described have been kept in a freezer for more than 2 years with no loss of activity.

Enzyme Assay. Enzyme reactions were carried out in single side-arm Warburg flasks at 37° C. To each flask were added 2 ml. of the enzyme preparation and 150 μg. of DDT in 30 μl. of ethanol or in 100 μl. of acetone. The volume was adjusted to 2.5 ml. with 0.1*M* tris(hydroxymethyl)aminomethane buffer, pH 8.8. Gassing of the reaction mixture with nitrogen for 20 minutes was carried out simultaneously with temperature equilibration of the flask. Introduction of 3 mg. of reduced glutathione dissolved in 0.5 ml. of 0.024*M* $NaHCO_3$ from the side-arm into the reaction mixture marked initiation of the assay. Reaction time was routinely set for 2 hours.

Extraction and Isolation of DDT Metabolites. Upon removal of the flask from the water bath, the reaction mixture was quantitatively transferred to a round-bottomed flask, an equal amount of *n*-hexane was added, and the flask was shaken mechanically for 1 hour. The mixture was centrifuged at 5000 × G for 10 minutes, and the two phases were separated. The aqueous and hexane portions were then analyzed for DDT and degradation products.

By using the colorimetric method of Schechter *et al.* (*22*), orienting tests invariably showed the presence of only unchanged DDT in the hexane portion whereas conjugated degradation products of DDT remained in the aqueous phase. Hence, in all subsequent tests only the aqueous portion was used for analysis.

The aqueous portion was evaporated to dryness, and the residue was reacted overnight with 10 ml. of formic acid and 10 ml. of 30% hydrogen peroxide. Following extraction with *n*-hexane, the acid was removed by vacuum distillation and the residue hydrolyzed with 6*N* HCl and methanol (1:1 by volume) for 18 hours. A second extraction with *n*-hexane followed, and the two extracts were then combined. This solution was transferred to a separatory funnel and shaken with 2% NaOH for several minutes. After separation of the two phases, the alkali was decanted, acidified with 6*N* HCl, and extracted with diethyl ether. This procedure enabled the separation of acidic and neutral components from the mixture.

Several authentic DDT derivatives such as the ethylene derivative DDE, the acidic product DDA, dichlorodiphenyl trichloroethanol, and 4,4-dichlorobenzophenone were subjected to the same treatment as described above for the DDT metabolites. No change was noted in these compounds due to oxidation with performic acid or hydrolysis with methanolic HCl.

Purification of extracts was accomplished by column chromatography through chromatographic grade silicic acid (*9*) using 1% ether in redistilled *n*-hexane as eluent for the neutral material and peroxide-free ether as eluent for the acidic metabolite. The neutral material was further chromatographed through activated alumina (*23*) to separate neutral components in a mixture.

Analytical Procedures. C^{14} was assayed in a liquid scintillation counter (Tracerlab LSC-10). Characterization and identification of breakdown products of DDT was accomplished by:

—Colorimetric analysis by the method of Schechter *et al.* (*22*);

—Ultraviolet absorption spectra, using a Cary Model 14 recording spectrophotometer;

—Infrared spectroscopy, using a Perkin-Elmer Model 21 spectrophotometer with NaCl optics;

—Reverse-phase paper chromatography by Mitchell's (*14*) and Winteringham's (*31*) methods, using aqueous and nonaqueous solvent systems. Cochromatography with authentic DDT derivatives was used to ascertain the identity and purity of metabolites;

—Bioassay of DDT metabolites for their insecticidal properties using third-instar *Aëdes aegypti* larvae. Acetone solutions of extracts in 1-ml. aliquots were added to glass bowls containing 100 ml. of water and 20 mosquito larvae per bowl. The mortalities at 24 hours were compared with those obtained by exposure of larvae to known concentrations of *p,p'*-DDT which were run concurrently with each unknown sample.

Results

Analysis by the colorimetric method of Schechter *et al.* (*22*) of neutral and acidic reaction products of DDT metabolism yielded pink-colored complexes with characteristic absorption maxima at 520 to 530 mμ. This method shows the transformation of DDT into various derivatives but does not further characterize or identify the breakdown products. The same characteristic curves were obtained with extracts of susceptible and DDT-resistant lice.

Following elution of neutral and acidic components through silicic acid and further separation of neutral material on activated alumina, at least three metabolites were obtained. These have been identified by paper chromatography as 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (DDE, metabolite A); 4,4-dichlorobenzophenone (DBP, me-

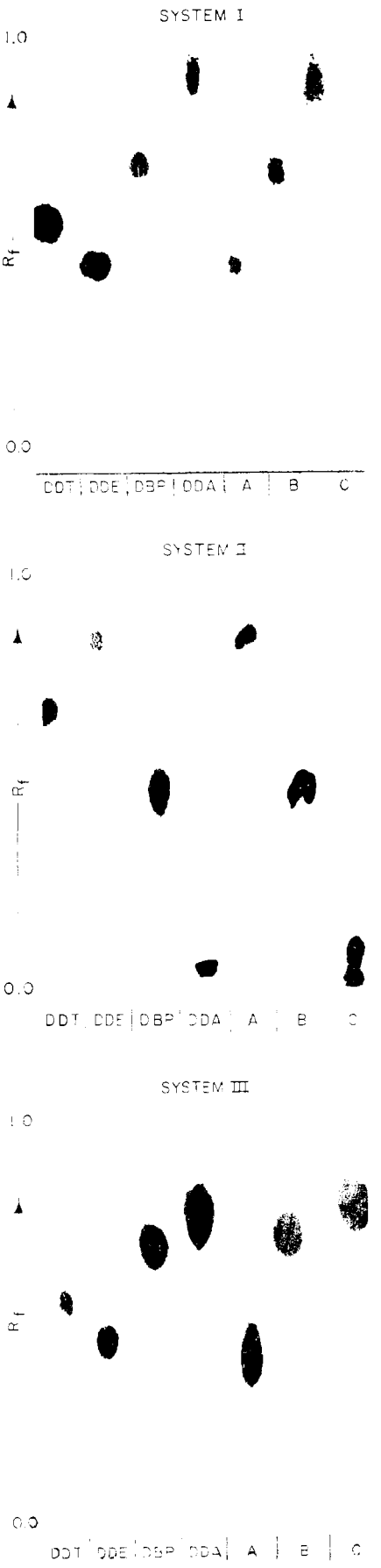


Figure 1. Autoradiochromatograms of DDT, DDE, DBP, DDA, and of metabolites obtained following incubation of louse homogenate with C^{14}-DDT

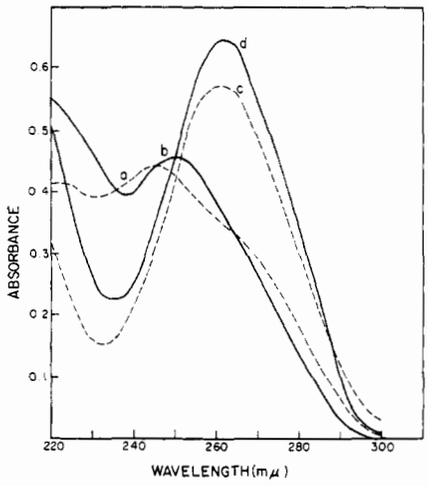
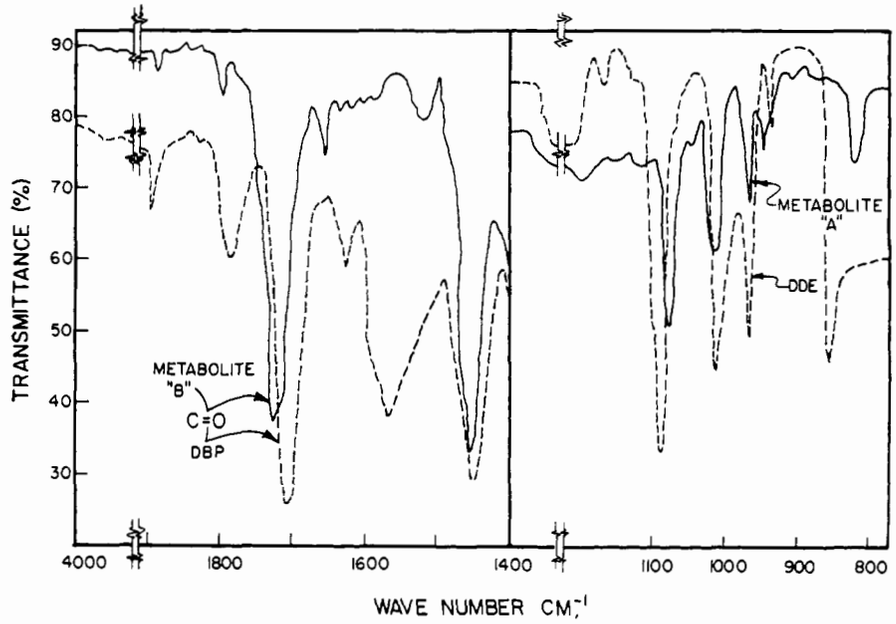


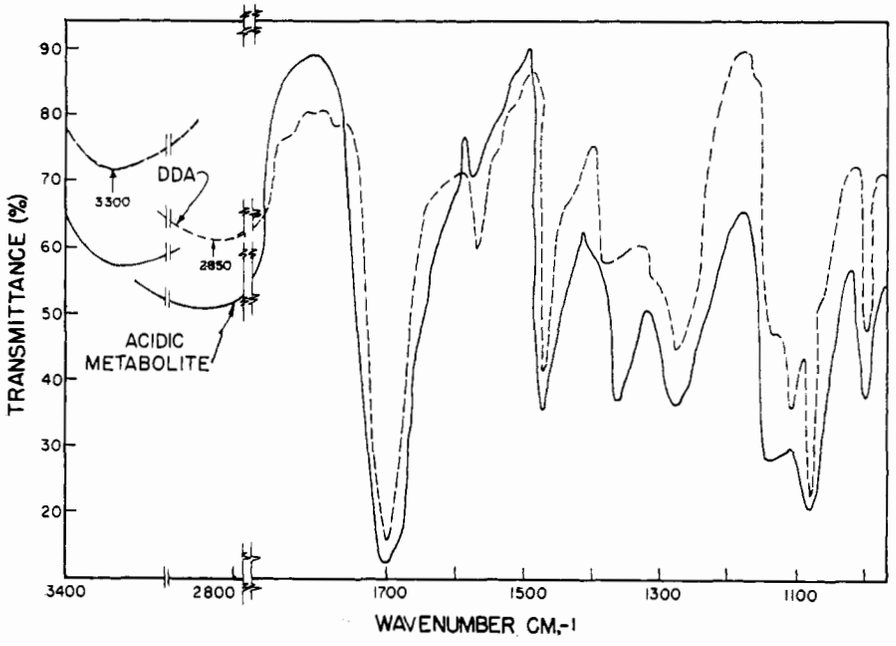
Figure 2. Ultraviolet absorption spectra for (*a*) DDE, (*b*) neutral metabolite A, (*c*) DBP, and (*d*) neutral metabolite B

Table I. R_f Values for DDT, DDE, DBP, DDA, and Metabolites A, B, and C[a]

Compound	R_f ± Std. Dev. System I[a]	System II[a]	System III[a]
DDT	0.550 ± 0.025	0.636 ± 0.030	0.615 ± 0.029
DDE	0.445 ± 0.007	0.873 ± 0.039	0.526 ± 0.023
DBP	0.651 ± 0.012	0.556 ± 0.037	0.722 ± 0.033
DDA	0.970 ± 0.017	0.067 ± 0.020	0.828 ± 0.034
Metabolite A	0.455 ± 0.024	0.870 ± 0.025	0.535 ± 0.023
Metabolite B	0.664 ± 0.032	0.505 ± 0.031	0.748 ± 0.014
Metabolite C	0.956 ± 0.032	0.058 ± 0.013	0.805 ± 0.034

	Stationary phase	Mobile phase
[a] System I:	4% mineral oil	acetone-water (3:1 v./v.)
System II:	35% dimethylformamide	isoöctane
System III:	4% mineral oil	ethanol–water–ammonium hydroxide (8.5:1:0.5 v./v.)





tabolite B); and bis-(*p*-chlorophenyl)-acetic acid (DDA, metabolite C).

Autoradiochromatograms and R_f values using three solvent systems are shown in Figure 1 and Table I, respectively.

Ultraviolet spectra of neutral metabolites A and B are shown in Figure 2. The absorption peak of metabolite A at 252 mμ is fairly close to that of authentic DDE at 247 mμ. The slight deviation might be due to the presence of impurities which are eluted with the metabolite through the alumina column. The spectrum of metabolite B with an absorption peak at 262 mμ is identical with that of authentic 4,4-dichlorobenzophenone.

So far, the authors have been unable to obtain a satisfactory ultraviolet spectrum for the acidic metabolite due to the presence of interfering glycerides which are difficult to remove.

Additional information on the identity of DDT metabolites is furnished by infrared analysis. Typical infrared spectrograms are shown in Figure 3. Neutral metabolite A shows very close similarity to the identifying DDE peak at 965 cm.$^{-1}$ A strong absorption peak at 1725 to 1700 cm.$^{-1}$, indicating the presence of a C=O group such as that obtained with 4,4-dichlorobenzophenone, is characteristic of neutral metabolite B. The acidic metabolite shows a major peak at 1700 cm.$^{-1}$ due to C=O absorption, and a broad absorption band over the range 3500 to 2500 cm.$^{-1}$ The latter is due to OH stretching vibration

◀ Figure 3. Infrared absorption spectra for (*upper*) 1% solutions in spectrograde CCl_4 of DDE, DBP, and neutral metabolites A and B, and (*lower*) 1% solutions in redistilled $CHCl_3$ of DDA and acidic metabolite C 1.0-mm. NaCl microcells

of the carboxyl group, and often is used as supporting evidence in characterizing carboxylic acids (*2*). These absorption regions coincide with the spectrum of DDA. In addition to the identifying peaks, all three metabolites possess peaks in common at 1480, 1390, 1090, and 1010 cm.$^{-1}$ which also are characteristic of DDE, DDA, and DBP.

Finally, bioassay results against mosquito larvae showed a marked loss in toxicity of all three metabolites, in line with the corresponding derivatives DDE, DDA, and DBP.

The quantitative aspects of DDT metabolism with purified enzyme preparations will be dealt with in another paper (*13*). In general, the distribution of recovered metabolites was in the order of 20% DDE, 40% DBP, and 40% DDA.

Pathway of DDT-Metabolism. A conspicuous feature of these results is the revelation that the DDT enzyme complex of louse homogenate consists of more than one enzyme entity. Since three metabolites were obtained, the obvious question is: What is the pathway of DDT metabolism?

To ascertain the sequence of steps involved in the degradation of DDT, enzyme assays were made with C^{14}-DDE, C^{14}-DDA, and C^{14}-DBP as substrates. By using identical assay conditions and the same criteria for identifying degradation products as those described in the text under Methods, the results were as follows:

—DDE is not metabolized and not conjugated and is recovered quantitatively in the hexane extract.

—DDA is metabolized to DBP. Both the unchanged portion of DDA and the metabolic product DBP are recovered in conjugated form from the aqueous phase.

—DBP is not metabolized and, like DDE, is recovered unconjugated in the hexane portion.

It is interesting to note that conjugation of the metabolites DDE and DBP occurred in the process of DDT-metabolism but not when authentic DDE and DBP were used as enzyme substrates. DDA was conjugated in both cases.

These results suggest that DDT metabolism proceeds according to the scheme shown in Figure 4, with the added assumption that other intermediate steps might be involved.

By analogy, metabolism of DDT in the American roach (*21*) and the Madeira roach (*10*) yields several polar metabolites in addition to DDE. One of the polar metabolites appears to be 4,4-dichlorobenzophenone (*8*). Later studies (*1*), however, imply that the latter metabolite is indeed 2,2-bis-(*p*-chlorophenyl) - 1,1,1 - trichloroethanol (commercially known as Kelthane). Tsukamoto (*28–30*) has shown that Kelthane is the principal metabolite

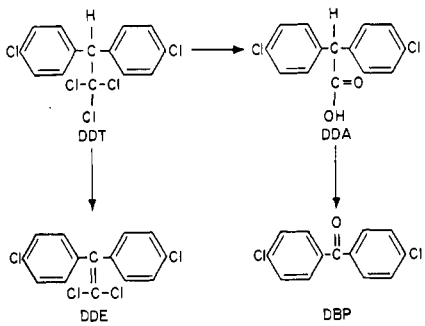


Figure 4. Pathway of in vitro DDT metabolism by enzymes of the body louse

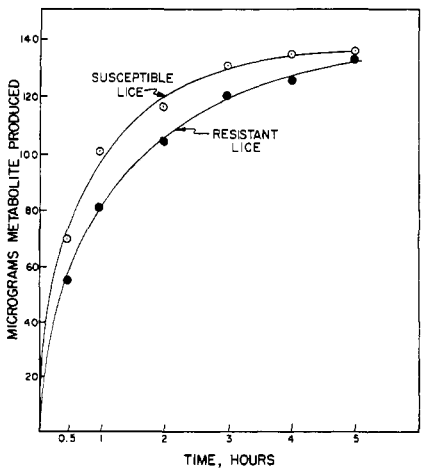


Figure 6. Effect of reaction time on DDT-ase activity of louse homogenate

Reaction mixture contained 2 ml. of homogenate, 0.5 ml. of tris buffer pH 8.8, 3 × 10^{-3} M reduced glutathione, 150 μg. of C^{14}-DDT; incubated at 37° C. for indicated time periods. Metabolites include DDE, DBP, and DDA

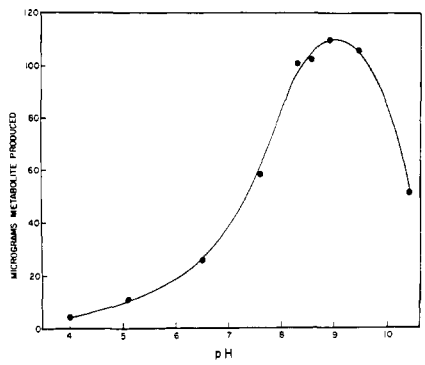


Figure 5. Effect of pH on DDT-ase activity of louse homogenate

Reaction mixture contained 2 ml. of homogenate, 0.5 ml. of buffer at indicated pH, 3 × 10^{-3} M reduced glutathione, 150 μg. of C^{14}-DDT; reaction time: 2 hours under N_2, at 37° C. Metabolites include DDE, DBP, and DDA

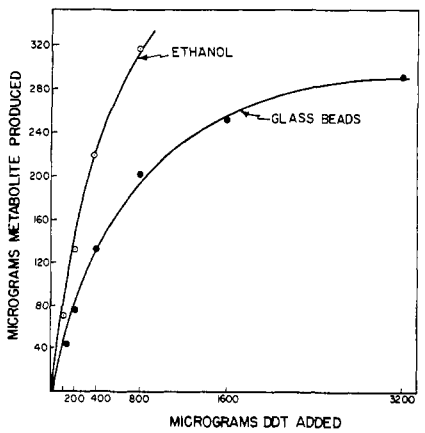


Figure 7. Effect of substrate concentration on DDT-ase activity of louse homogenate

Reaction mixture contained 2 ml. of homogenate, 0.5 ml. of tris buffer pH 8.8, 3 × 10^{-3} M reduced glutathione, varying amounts of C^{14}-DDT precipitated on glass beads or dissolved in ethanol; reaction time: 2 hours under N_2, at 37° C. Metabolites include DDE, DBP, and DDA

resulting from the degradation of DDT by the vinegar fly *Drosophila melanogaster*, and suggested that this metabolite might be the product of DDT metabolism in other insects as well. This possibility was taken into account in the present study. Using the Fujiwara test for the detection of Kelthane (*28*), the authors were unable to show the presence of this metabolite as a product of DDT metabolism in louse homogenates.

Characteristics of the Enzyme System

Sternburg *et al.* (*24*) described the properties of the enzyme DDT-dehydrochlorinase which catalyzes the breakdown of DDT in houseflies. The authors emphasized the complexity of factors involved in studying the kinetics of the reaction due to the extreme insolubility of the substrate DDT in the aqueous medium. These limitations are not of vital importance for assay of the louse enzyme since the latter can tolerate fair quantities of acetone or alcohol which form stable suspensions with the substrate. Also, the addition of 3 mg. of pancreatin to the assay medium prevents lumping of particulate matter, thus resulting in a more homogeneous system.

The elegant spectrophotometric assay system using lipoprotein as a solubilizing agent for DDT (*11*) could not be used here because louse homogenate contains more than one DDT enzyme as evidenced by the recovery of three metabolites. Furthermore, the dark brown color which is characteristic of louse homogenates precludes the use of a spectrophotometric method.

Like most enzyme systems, the louse DDT-ase complex has a narrow pH range through which it exerts its maximum effect. Optimum activity is at pH 8.8 to 9.0 (Figure 5). It is almost completely inhibited at pH 3.5 to 4.0

Table II. Effect of Cofactors, Nitrogen Gas, and DDT-Synergists on Rate of in vitro Breakdown of C^{14}-DDT by Body Louse Homogenates

(Assay conditions as described in text)

Homogenate	Cofactor	N_2	DDT Synergist, 150 μg.	DDT Metabolized, μg.
+	Glutathione	+	...	123
+	+	−	...	68
+	−	+	...	11
+	−	−	...	5
−	+	+	...	0
+	L-Cysteine	+	...	126
+	Thioglycollate	+	...	121
+	Ascorbate	+	...	113
+	Coenzyme A	+	...	105
+	Glutathione	+	DMC[a]	112
+	+	+	MR-60[b]	119
+	+	+	BST[c]	120

[a] Bis-(*p*-chlorophenyl)methyl carbinol.
[b] Bis-(*p*-chlorophenyl)chloromethane.
[c] *p*-Bromobenzenesulfonotoluidide.

and shows diminished activity above pH 9.5.

The rate of enzyme reaction varies with the type of preparation. Homogenates used throughout this work containing 100 lice, or 2.1 mg. of protein N per ml., sustain a fairly linear reaction rate for the first hour, after which the reaction velocity diminishes with time (Figure 6). The reaction rate was similar for homogenates of susceptible and DDT-resistant lice.

The effect of substrate concentration on DDT metabolism is shown in Figure 7. The reaction velocity was lower when DDT was applied on glass beads (*24*) than when dissolved in ethanol or acetone. With ethanol solutions, the optimum concentration of DDT appears to be 800 μg. under the assay conditions. This optimum concentration might be the result of the limited solubility of DDT in ethanol (2% at room temperature).

Unlike most known enzyme systems, including housefly DDT-dehydrochlorinase, the activity of louse DDT-ase increases with a rise in temperature up to 50° C., the highest temperature tried. The enzyme has a Q_{10} of approximately 2 between 30° and 40° C. This is not surprising, for the unique characteristic of this enzyme system is its stability to heat. On numerous occasions, homogenates were boiled under reflux for 1 hour or longer with no deleterious effect. On the contrary, a slight increase in activity was noted after heating.

Maximum activity can be maintained only in the presence of a cofactor with reducing properties. The most effective cofactor is reduced glutathione. However, cysteine, thioglycollate, coenzyme A, and ascorbate can replace glutathione at a slightly higher concentration. The fact that ascorbate can activate the enzyme indicates that the system is not dependent on exogenous sulfhydryl groups. Temporary or continuous gassing with nitrogen is essential for maintaining anaerobic conditions. The effects of cofactors and nitrogen gas on enzyme activity are shown in Table II.

Effect of DDT Synergists on Enzyme Activity. A characteristic feature of so-called DDT synergists is their ability to block the dehydrochlorination of DDT to DDE by competitive action either in vivo (*20*) or in vitro (*16*). It has previously been demonstrated that several DDT synergists had no effect on mortality of DDT-resistant lice (*7*) or on the in vivo metabolism of DDT by resistant and susceptible lice (*19*). Similar results have been obtained in vitro with louse homogenates (Table II). It is possible that the action of these synergists is specific for dehydrochlorinase enzymes which catalyze the breakdown of DDT to DDE, and have slight or no effect on enzyme systems which catalyze the degradation of DDT to other metabolites. Since DDE forms only a small fraction of the total metabolic pool in louse homogenates (the yields of DDA and 4,4-dichlorobenzophenone being much higher), specificity for dehydrochlorinase enzymes might explain why such synergists are ineffective against body lice.

Comparative Aspects. A comparison of optimum conditions for enzyme activity between housefly DDT-dehydrochlorinase (*24*) and body louse DDT-ase reveals that both systems require reduced cofactors for enzyme activation, although louse DDT-ase is not specific for either glutathione or a sulfhydryl group. Other differences include heat stability, pH optimum, temperature range, and the ineffectiveness of DDT synergists to block the degradation of DDT by louse DDT-ase.

These unique characteristics must give impetus for further inquiry into the fundamental properties and the kinetics of purified preparations of this enzyme system.

Discussion

With regard to the mechanism of louse resistance to DDT, admittedly more questions have been raised than answers given. Both susceptible and DDT-resistant lice metabolize DDT in vitro at an approximately equal rate and yield the same metabolites. Yet, DDT-metabolism in vivo occurs only in the resistant strain.

This situation is unlike that found in DDT resistant houseflies which contain a much higher dehydrochlorinase titer than susceptible flies, and in which DDT-metabolism can be correlated with enzyme content and degree of survival.

In these circumstances, one might ask if DDT metabolism by the resistant louse is in any way related to resistance. Except for possible permeability or solubility factors, the insect must detoxify DDT in order to survive. Hence, detoxication of the insecticide is an active mechanism of resistance, but the reason for the failure of the detoxifying enzymes to afford protection to the susceptible louse is not understood.

At present, one can only speculate on the cause of DDT resistance in the louse such as: local detoxication at a vital site might favor the resistant strain; an effective nerve barrier in the resistant strain might prevent DDT from penetrating sensitive neurones; differences in lipoid content and composition might affect the solubility of DDT within tissues; and a combination of these and other unknown factors may operate.

Lack of information on these and other related biochemical phenomena tend to accentuate the fact that we are still in ignorance about the physiological basis of DDT resistance in body lice.

Acknowledgment

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PLANT SUSCEPTIBILITY TO INSECTS

Chemical Factors Influencing Host Selection by the Mexican Bean Beetle *Epilachna varivestis* Muls

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Studies of extracts of the seeds of *Phaseolus vulgaris* (L) indicated a factor or factors influencing feeding behavior of the Mexican bean beetle (*Epilachna varivestis* Muls). Sucrose was isolated and identified and shown to be the factor responsible for selectivity in the bioassay used. Quantitative sugar determinations on the seeds of a series of resistant and nonresistant plants showed that the concentration of nonreducing sugars is significantly higher in the seeds of nonresistant plants. Sucrose concentration in seeds is suggested as an index of resistance to attack by the Mexican bean beetle. In addition, the importance of sucrose in bioassay involving feeding behavior is discussed.

CERTAIN VARIETIES of bean plants are more susceptible to attack by the Mexican bean beetle than are others. The problem of determining the reason for this susceptibility may be approached from a number of points of view. If a particular variety of plant is preferentially attacked by insects, it may be that this variety possesses a factor which attracts the insect to the plant. Many host-plant relationships have been investigated from this point of view and, in several cases, chemicals have been isolated which function as attractants (*6–11,16,17*). On the other hand, the lack of a repellant or resistance factor in susceptible plants may be involved in determining host-plant specificity (*12–15*). The authors decided to look for a chemical factor that would act as an attractant for these insects, and that would be present only in the susceptible varieties.

Leaf material from *Phaseolus vulgaris* (L) was lyophilized and extracted with a series of solvents of varying polarity. These extracts were then tested by saturating filter paper disks with the extract and exposing these disks, along with control disks containing no extracted material, to the beetles. Details of this procedure have been reported elsewhere (*5*).

The criterion of attractiveness was the number of feeding marks or ridges the beetles made on the filter paper disks.

Aqueous or alcoholic extracts of either leaves or seeds gave a positive response in the bioassay procedure. Since seeds are easier to store and extract, and since bioassay indicated activity in this plant portion, seeds were used for the investigations.

The alcoholic extract from a 50-gram sample of ground seeds was filtered and the solvent removed. The dried residue was then dissolved in distilled water. A portion of this aqueous solution, after being heated on a water bath for 30 minutes, gave a negative Benedict's test for reducing sugars. The remaining sample was made acidic (pH 4.5) with dilute HCl and subjected to the same treatment. After neutralization of the solution, a positive Benedict's test was obtained.