THE ABSORPTION AND METABOLISM OF [^{14}C]DDT IN DDT-RESISTANT AND SUSCEPTIBLE STRAINS OF THE CATTLE TICK *BOOPHILUS MICROPLUS*

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Summary

Engorged female cattle ticks of a DDT-resistant strain absorbed [^{14}C]DDT at a rate similar to females of a DDT-susceptible strain, but larvae of the resistant strain absorbed it at a faster rate than susceptible larvae.

In a chromatographic analysis of the metabolites of [^{14}C]DDT produced in larvae, at least 17 metabolites were isolated from larvae of each strain, and the corresponding metabolites from each strain were similar with regard to chromatographic behaviour, and for those of which spectra were obtained, to spectral characteristics as well. Infrared and ultraviolet spectra were obtained from nine metabolites and, of these, three were identified as DDE [1,1-dichloro-2,2-di(4-chlorophenyl)ethylene], dicofol [2,2,2-trichloro-1,1-di(4-chlorophenyl)ethanol], and 4,4′-dichlorobenzophenone. From spectral and chemical evidence the remaining six metabolites were classified as phenols (of possibly DDT, DDE, and dichlorobenzophenone) and an aromatic carboxylic acid.

DDE was the only metabolite detected in engorged female ticks and both strains produced similar amounts.

Larvae of both strains metabolized a greater proportion of a low dose than of a high dose. More of a low dose was metabolized by susceptible larvae than by resistant larvae. DDE, dicofol, 4,4′-dichlorobenzophenone, and some unknown metabolites were considerably less toxic than DDT to susceptible larvae.

It was concluded that in cattle ticks there was no causal relation between resistance to DDT and either absorption or metabolism.

I. INTRODUCTION

Comparative studies of the metabolic fate of DDT in resistant and susceptible arthropods have yielded valuable information on the role of detoxification mechanisms in resistance. A convincing case can be argued from the available data (Sternburg, Kearns, and Bruce 1950; Perry and Hoskins 1951) that in houseflies the ability of resistant strains to convert DDT to DDE [1,1-dichloro-2,2-di(4-chlorophenyl)ethylene] at a faster rate than susceptible strains is causally related to resistance. Although a similar ability is possessed by some resistant strains of *Aedes aegypti*, a causal relationship between the degree of dehydrochlorination and resistance is not clear (Chattoraj and Brown 1960). Detoxification by hydroxylation has been studied in many pests and dicofol [2,2,2-trichloro-1,1-di(4-chlorophenyl)ethanol] has been found as a major metabolite in *Blatella germanica* (Hoskins, Miskus, and Eldefrawi 1958), *Drosophila melanogaster* (Tsukamoto 1959), *Musca domestica* and *Phormia regina* (Schonbrod, Philleo, and Terriere 1965), and in *Triatoma infestans* (Agosin, Morello, and Scaramelli

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1964). It is only in the latter pest that hydroxylation has been proposed as a causative resistance mechanism (Morello 1964).

Some DDT-resistant strains of arthropods metabolize DDT at the same rate as susceptible strains, indicating that there is no metabolic basis for resistance in these strains. For instance no correlation could be found between the rates of metabolism of DDT and survival of DDT-resistant strains of *Anthonomus grandis* (Blum, Earle, and Roussel 1959) and *Pediculus humanus* (Perry, Muller, and Buckner 1963).

Resistance of cattle ticks to DDT was recognized in Australia in 1955 (Legg, Brooks, and Joyner 1955; Stone 1957) and the present study forms part of an attempt to discover the mechanism of resistance of this arthropod to DDT.

II. Materials and Methods

(*a*) *Ticks*

The DDT-resistant strain (R) used was that described by Stone (1957) who cultured the strain in this laboratory under selection pressure. The proportion of resistant individuals in the strain did not fluctuate markedly and at all times was never less than 95% (Stone, unpublished data). The reference susceptible strain (S) was cultured under the same conditions as the resistant strain. Larvae used for experiments varied in age from 10 to 21 days. In any one experiment S and R larvae were of the same age. Concentration-response tests utilizing a dipping technique showed that R larvae were 8·8 times more resistant to DDT than S larvae (Schnitzerling, unpublished data).

(*b*) *Radioactive Compounds*

Uniformly labelled [^{14}C]1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane* ([^{14}C]DDT, specific activity 12·2 mCi/g) was obtained in radiochemical purity by paper (Mitchell 1956) and column (Schuntner and Schnitzerling 1965) chromatography. Chemical purity was established by infrared spectroscopy. Chemically pure [^{14}C]1,1-dichloro-2,2-di(4-chlorophenyl)ethylene ([^{14}C]DDE) was obtained by similar means after preparations from [^{14}C]DDT by dehydrochlorination.

(*c*) *Non-radioactive Compounds*

Samples of these were obtained as gifts, as reagent grade chemicals, and by laboratory synthesis. The purity of laboratory-synthesized compounds was established from the melting point and infrared spectroscopy. Compounds used included the following:

1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane(DDT)
1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane
1,1-dichloro-2,2-di(4-chlorophenyl)ethylene(DDE)
1,1-dichloro-2,2-di(4-chlorophenyl)ethane†
2,2,2-tricholoro-1,1-di(4-chlorophenyl)ethanol (dicofol)†
1,1,1-trichloro-2,2-di(4-methoxyphenyl)ethane‡
1,1-dichloro-2,2-di(4-methoxyphenyl)ethylene
1-hydroxy-2,2-di(4-chlorophenyl)ethane†
2,2-di(4-chlorophenyl)ethylene
2,2-di(4-chlorophenyl)acetic acid, methyl and ethyl esters
1,1-diphenyl ethane
4,4′-dichlorobenzophenone(DBP)
4-chlorophenol
4-hydroxyacetophenone
4-methoxyacetophenone
4-chlorobenzoic acid
phenol

* Supplied by the World Health Organization, Geneva.
† Supplied by Rohm and Hass Company, Philadelphia, Pennsylvania 19105.
‡ Supplied by Geigy (A'asia) Pty. Ltd., Botany, N.S.W.

Infrared and ultraviolet spectra were obtained for these compounds and these were used to aid the interpretation of spectra of unknown metabolites.

(*d*) *Treatment of Ticks*

For all experiments [^{14}C]DDT was diluted with DDT 1 : 10. Larvae were treated by dipping in [^{14}C]DDT colloids prepared according to the method of Roulston, Schuntner, and Schnitzerling (1966). Engorged female ticks were treated by topical dosing of their dorsal surface using a microsyringe. Numbers of females per dosage ranged from 5 to 50. Larvae and females were incubated at 30°C and high humidity.

(*e*) *Extraction of Radioactive Compounds*

For absorption studies treated larvae and engorged female ticks were washed with acetone to extract "external" radioactive compounds. For comparative metabolic studies treated ticks were not washed. "Internal" or "total" radioactive compounds were extracted from larvae by homogenization with methyl cyanide in an all-glass apparatus and from engorged females by maceration at high speed in acetone. Extracts were spotted on paper or applied to columns for chromatographic separation, or evaporated on planchets for radioactivity counting.

For detailed metabolic studies on large samples of larvae as shown in Figure 1 total radioactive compounds were extracted by homogenization of larvae with methyl cyanide in a reciprocating steel ball mill. The solvent phase was subjected to further extraction prior to column chromatography as shown in Figure 1.

(*f*) *Separation and Purification of Metabolites*

Preliminary work indicated that ticks produced a large number of metabolites of DDT. Investigations of several paper-chromatographic systems showed that none would resolve more than five fractions. The phenoxyethanol and soya bean oil systems of Mitchell (1956 and 1957) gave reproducible results and satisfactorily resolved DDT and DDE from one another and from all other extracted metabolites. These two systems were used in comparative studies of the production of DDE and "metabolites other than DDE" in R and S larvae and engorged female ticks.

The method which gave a satisfactory separation of the metabolites involved liquid–liquid extraction followed by column chromatography similar to that devised for candidate metabolites of DDT by Schuntner and Schnitzerling (1965). Doses of DDT compatible with negligible mortality in both strains of larvae after 96 hr incubation were 240 and 30 μg per gram for R and S larvae respectively. A high production of metabolites was desirable since it was anticipated that purification of the fractions obtained from the initial chromatography would incur considerable losses of metabolites. Accordingly 100 g of R larvae were dosed as indicated above and the metabolites were separated as outlined in "Separation of metabolites of R larvae", Figure 1. The fractions from columns 1, 2, and 3 were subjected to further column chromatography using different systems, similar ones to those described by Schuntner and Schnitzerling (1965). Frequently the ultraviolet spectrum of an impurity in a fraction differed markedly from that of the metabolite. In most cases there was a partial resolution of the metabolite from the impurity wherein the distinctive spectrum of a metabolite could be correlated with the amount of radioactivity. By this means fractions of higher purity were selected and if necessary chromatographed further. Final evidence for the purity of a metabolite was an infrared spectrum which remained unaltered on chromatography of the metabolite in different systems. Later, a comparison was made of metabolite production in larvae of both strains under identical conditions of extraction and chromatography (Fig. 1). The elution conditions differed from those used previously but they resulted in improved resolution of fractions.

(*g*) *Counting and Scanning of Radioactive Compounds*

The radioactivity on paper chromatograms was recorded automatically after scanning with a thin end window Geiger–Müller tube. The radioactivity in fractions of column effluents or in "external" and "internal" extracts was determined by counting an evaporated aliquot in a scaler with a Geiger–Müller tube.

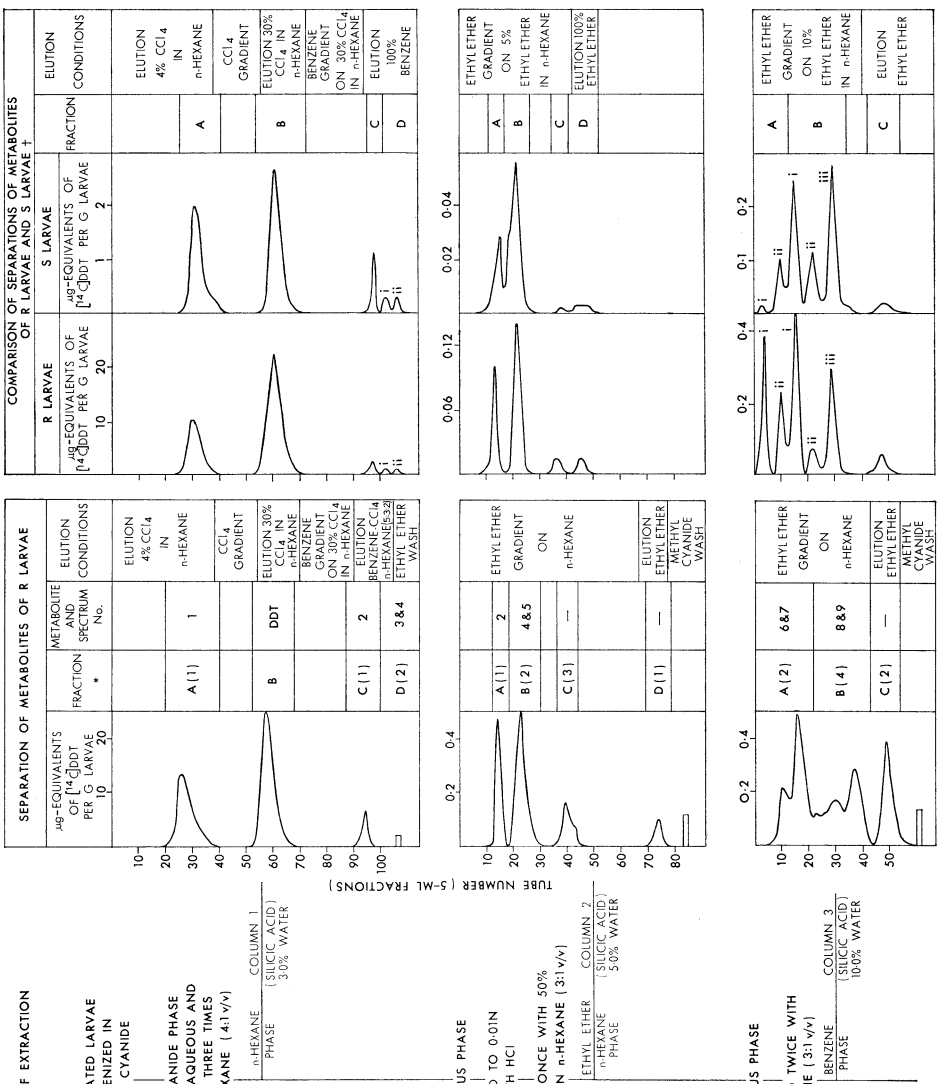

Fig. 1.—Liquid–liquid extraction and chromatographic separation of metabolites of DDT in R and S cattle tick larvae.
* The number in brackets refers to the total number of different metabolites resolved from each fraction.
† Roman numerals refer to subfractions which are shown in Table 4.

(*h*) *Chemical Analysis*

DDT was used in some absorption experiments with engorged female ticks and was determined in external and internal extracts by the method of Schechter *et al.* (1945).

(*i*) *Spectroscopy*

Infrared and ultraviolet spectroscopy of samples from columns were carried out using the potassium bromide pellet technique for the former and solutions in n-hexane and aqueous alcohols for the latter method.

III. Results

(*a*) *Recovery of Radioactive Compounds*

The equivalent of 97% of the applied dose was recovered in the solvent extracts. Approximately 3% of the absorbed dose remained in the solid residue and this was difficult to extract. An ethanol extract of the acidified residue contained approximately equal quantities of DDT and highly polar metabolites. In all experiments the solid residues from the initial extraction were discarded without further treatment. Approximately 95% of radioactive compounds was recovered from columns 1, 2, and 3 (Fig. 1) and paper chromatograms.

(*b*) *Absorption*

The rates of absorption of [^{14}C]DDT or DDT were similar in R and S engorged female ticks and varied similarly according to the solvent used (Table 1). Comparison of rates of absorption of [^{14}C]DDT in larvae (Table 2) is not simple. It was found that DDE was excreted by larvae of both strains and there was no effective way of quantitatively separating larvae and excreta. However, the amount was small and even after 72 hr of incubation probably did not exceed 10% of the applied dose. Further, it was quite likely that some internal DDT and DDE were removed in the external wash as some polar metabolites which were not excreted were removed also. Attempts to prevent the elution of internal DDT and metabolites by the external wash using different solvent washes such as aqueous methyl cyanide, acetone, and Triton X-100 solutions and n-hexane were unsuccessful as all the washes contained polar metabolites. Thus the rates of absorption as they appear in Table 2 represent relative values only. However, they indicate that R larvae absorbed more DDT and at a faster rate than S larvae.

(*c*) *Characterization of Metabolites*

The initial separation of metabolites of R larvae and the treatment employed are shown in Figure 1. There was a coincidence of peak–height positions of corresponding fractions for all three column separations. Infrared spectra verified the chemical similarity of all corresponding R and S fractions separated in column 1. Identical behaviour was observed for all corresponding R and S fractions from columns 2 and 3, when they were subjected to further chromatography. Some of these fractions yielded more than one metabolite. When these were taken into account it was found that at least 17 metabolites of DDT were produced in R and S larvae.

Spectra were obtained for nine of the more abundant metabolites. These are shown in Figures 2 and 3. The agreement between the spectra of metabolites 1, 2,

and 3 and authentic compounds confirms their identity as DDE, dicofol, and DBP respectively.

TABLE 1

COMPARISON OF ABSORPTION OF TOPICALLY APPLIED [^{14}C]DDT AND DDT IN R AND S ENGORGED FEMALE TICKS

Dose (μg/tick)	Solvent (1 μl)	Incubation Time (hr)	% of Dose Absorbed	
			R Strain	S Strain
[^{14}C]DDT				
50	1 : 1 Amyl alcohol–methoxytriglycol	24	2·0	2·7
50		48	3·6	3·2
50		72	2·9	3·4
DDT				
14	Peanut oil	72	2·5	2·8
28		72	3·1	1·8
39		72	4·4	2·3
58		72	4·1	4·9
14		144	12·8	11·3
28		144	10·6	9·2
39		144	11·6	11·0

A search of the spectral literature and our own collection of spectra of some analogues of DDT failed to find matching spectra for the remaining six metabolites. A comparative study of their spectra and those of the analogues of DDT was made, and broad structural classifications were assigned to them on this basis. Also, supporting evidence for the assignment of a phenolic character to all the metabolites except 8 was provided in part by the chemical and spectral properties of metabolite 4.

TABLE 2

COMPARISON OF ABSORPTION OF [^{14}C]DDT IN R AND S LARVAL TICKS

Incubation Time (hr)	% of Dose Absorbed		Incubation Time (hr)	% of Dose Absorbed	
	R Strain	S Strain		R Strain	S Strain
30 μg [^{14}C]DDT/g larvae			240 μg [^{14}C]DDT/g larvae		
16	68·0	47·5			
24	74·4	61·0	24	74·4	—
48	83·6	62·7	48	74·5	—
72	81·4	55·0	72	79·8	—
96	80·2	53·0	96	71·9	—

The spectral study revealed the possible presence in these metabolites of the skeletal structure of DDT in 5, 6, 7, and 9, an aromatic structure in 8, a hydroxyl group in 5, 6, 7, 8, and 9, and a carbonyl group in 8 and 9. Metabolite 8 is most likely

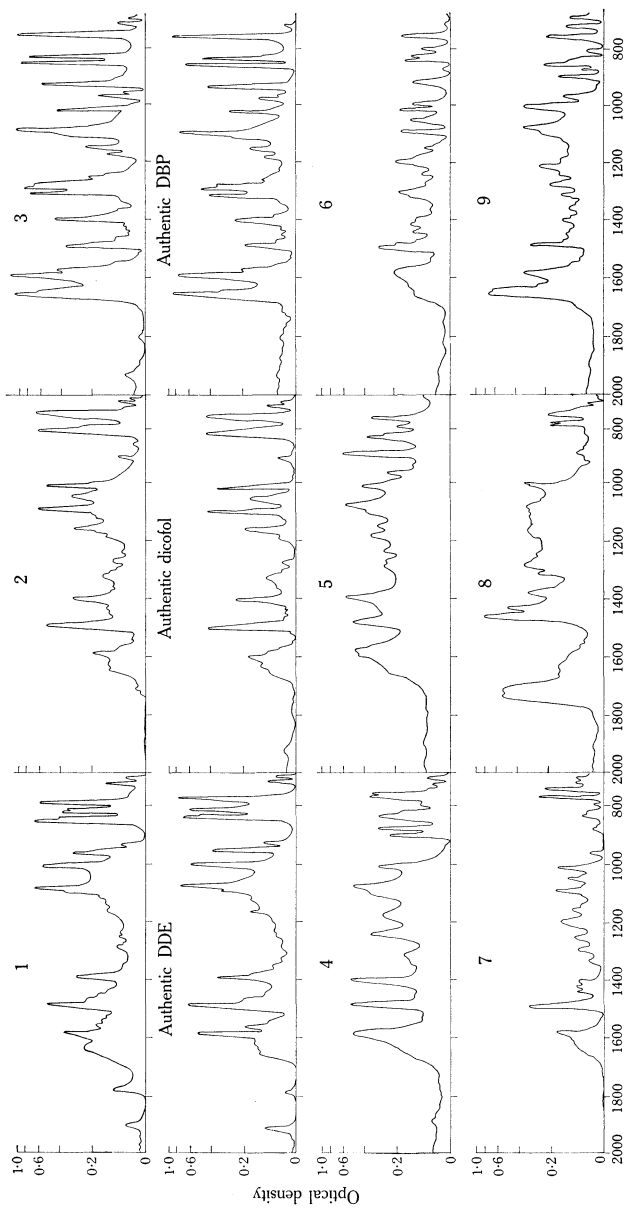

Fig. 2.—Infrared spectra of some DDT metabolites recovered from R cattle tick larvae and some authentic compounds.

acidic, because prior to final purification and spectroscopy, it was extracted into dilute aqueous sodium bicarbonate from n-hexane.

Metabolite 4 would not extract into dilute aqueous sodium bicarbonate from n-hexane. Its ultraviolet spectrum in n-hexane showed two small peaks centred at 277 and 284 nm. A shift occurred in the general band centre of these peaks from 282 to 307 nm on changing from n-hexane to alkaline aqueous isopropanol solution and this was reversible on re-extraction from the acidified aqueous isopropanol solution. These phenomena are consistent with the behaviour of phenols (West 1956). Thus there seems little doubt that ring hydroxylation is a pathway of metabolism of DDT in ticks.

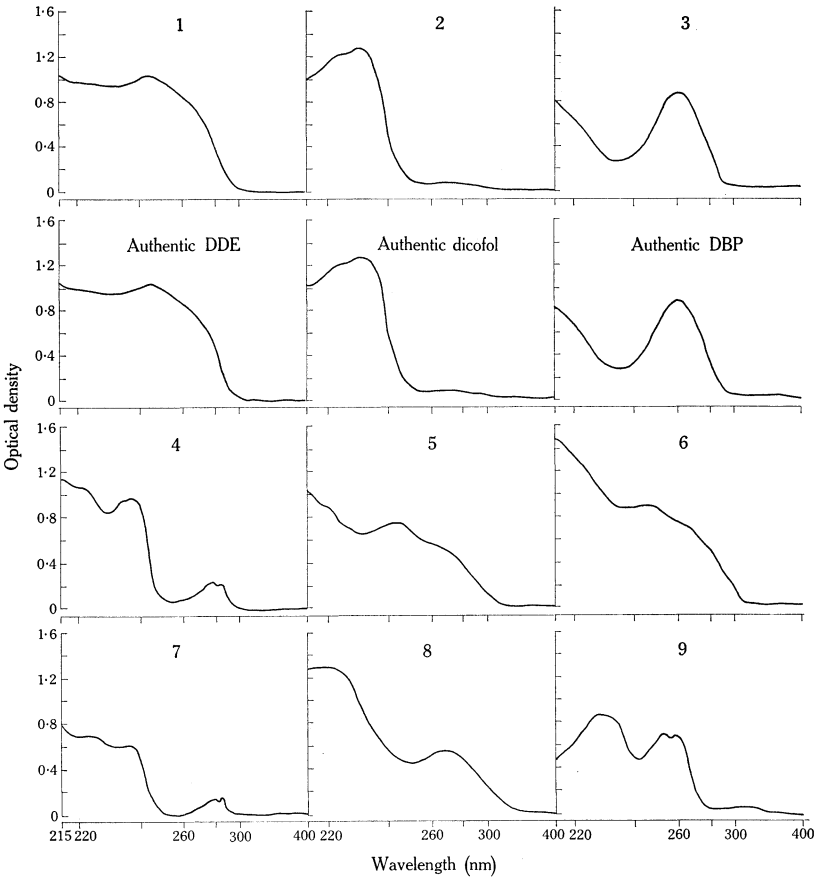

Fig. 3.—Ultraviolet spectra of some DDT metabolites recovered from R cattle tick larvae and some authentic compounds.

The ultraviolet spectra of metabolite 4 and 7 are similar and by analogy metabolite 7 could be a phenolic compound. The evidence supports the possibility that these metabolites could have either phenolic DDT or dicofol-type structures. However, a necessary requirement for either of these structures would be a

trichloroethane moiety considerably less liable to dehydrochlorination than DDT. Such could have been effected by *ortho*-substitution (Sternburg, Kearns, and Moorefield 1954) but this could not be proved by the evidence available.

The ultraviolet spectra of metabolites 5 and 6 are similar to that of DDE and by anology could have DDE-type structures.

On the basis of the foregoing evidence the metabolites are designated as the structural types listed in the following tabulation:

Metabolite No.	Structural Type
4 and 7	Phenolic DDT or dicofol
5 and 6	Phenolic DDE
9	Phenolic DBP
8	Aromatic carboxylic acid with an aliphatic moiety

Only one metabolite was detected in R and S engorged female ticks and its identity was confirmed as DDE by infrared spectroscopy.

(*d*) *Metabolism*

The results in the following tabulation show that when R and S engorged female ticks were given equal sublethal doses of 50 μg [^{14}C]DDT (applied topically in 1 μl of 1 : 1 amyl alcohol–methoxytriglycol) there was no difference between strains in the amounts of DDE found:

Incubation Time (hr)	DDE (μg-equiv/tick)	
	R Strain	S Strain
24	0·09, 0·11	0·08, 0·12
48	0·17, 0·21	0·16, 0·18
72	0·15, 0·23	0·17, 0·21

These determinations were made on two unpaired replicate samples of 15 engorged female ticks. Metabolites other than DDE were not detected for any of the incubation periods.

From Table 3 it can be seen that when R and S larvae were given sublethal doses of 30 or 77 μg [^{14}C]DDT per gram of larvae, more DDE was found in S larvae than in R larvae at the lower dose, and similar amounts were found in larvae of both strains at the higher dose. Under the same conditions S larvae produced slightly more metabolites other than DDE than R larvae. However, the percentage of DDT metabolized was lower at the higher dose than at the lower dose for both strains of larvae. Larvae of both strains were given a dose of 204 μg DDT per gram of larvae which was sublethal to R larvae but lethal to S larvae in less than 24 hr. At this dosage there was no difference between strains in the amounts produced of either DDE or metabolites other than DDE.

The amounts of the various fractions in metabolites other than DDE are summarized in Table 4. With one exception the amounts of metabolites produced were all greater in R larvae than in S larvae.

DDE, dicofol, DBP, and mixed components of fraction B (column 3, Fig. 1) were tested for toxicity against S larvae. All were considerably less toxic than DDT.

IV. Discussion

Resistant and susceptible engorged female ticks absorbed DDT at a similar rate but in larvae the rate was faster in the resistant strain than in the susceptible strain. This latter phenomenon has been observed previously in some strains of houseflies (Perry and Sacktor 1955; Wiesmann 1955) and may reflect strain differences in cuticle composition which are quite unrelated to specific DDT resistance. A slower rate of excretion of DDE by larvae of the resistant strain would have resulted in a spuriously faster rate of absorption of DDT for that strain. However, the amount of DDE excreted in either strain was small and even if there had been any difference

Table 3

[^{14}C]DDE and "metabolites other than DDE" in R and S larval ticks as determined by paper chromatography

Determinations made on two unpaired replicate 0·2-g samples of R and S larvae

Strain	Dose of [^{14}C]DDT (μg/g larvae)	Incubation Time (hr)	Amount of DDE Detected (μg-equiv/g larvae)	(%)*	Other Metabolites Detected (μg-equiv/g larvae)	(%)*
R	30 ‡	24	2·6, 3·2	8·3, 10·7	1·6, 2·0	5·3, 6·7
S	30 ‡	24	4·1, 4·9	13·8, 16·4	1·9, 2·1	6·4, 7·0
S†	30 ‡	96	11·0	36·6	5·9	19·6
R	77 §	24	4·0, 4·6	5·2, 6·0	2·0, 2·4	2·6, 3·2
S	77 §	24	4·2, 5·0	5·5, 6·5	2·9, 3·5	3·8, 4·6
R	77 §	72	12·2, 14·2	15·9, 18·5	8·0, 9·4	10·4, 12·2
R	204 ‖	4	9·1, 10·5	4·5, 5·1	1·4, 1·8	0·7, 0·9
S	204 ‖	4	8·6, 9·8	4·2, 4·8	1·5, 1·7	0·7, 0·9
R†	240¶	96	73·1	30·5	14·4	6·0

* As percentage of total radioactive compounds extracted.

† Metabolites determined by column chromatography as shown in Figure 1 (single determinations on 26 g of S larvae and 8 g of R larvae).

‡ Sublethal for both R and S strains.

§ Sublethal for R strain; lethal for S strain between 24 and 72 hr.

‖ Sublethal for R strain; lethal for S strain between 4 and 24 hr.

¶ Sublethal for R strain.

between strains in the rate of excretion of DDE it would have contributed but little to the difference found in the rates of absorption of DDT. Absorption was fastest in larvae of both strains during the 24-hr period immediately following dosing but thereafter was marked by considerable fluctuations. Metabolites were always found in external washes. It was possible that the fluctuations were caused by changing equilibrium conditions in respect to metabolite composition in the region of the cuticle. Larvae of either strain absorbed DDT at a much faster rate than engorged female ticks and this may in part contribute to the greater relative susceptibility of larvae to DDT (Stone 1957). However, there was no difference in the rates of absorption of DDT in resistant and susceptible ticks which could have contributed to survival of the resistant strain.

In a review by Perry (1964), it is clear that in some strains of houseflies resistance is due to the ability of resistant flies to metabolize DDT to DDE at a faster rate than susceptible flies. Although the presence of DDE was confirmed in both strains of ticks there was no difference between rates of conversion that could have caused resistance.

TABLE 4

COMPOSITION OF "METABOLITES OTHER THAN DDE" IN R AND S LARVAL TICKS 96 HR AFTER DOSING WITH 240 AND 30 μG [^{14}C]DDT/GRAM RESPECTIVELY

Column Fraction†	Amount of Metabolite (μg-equiv. [^{14}C]DDT/g larvae)		Identity or Type of Metabolite‡
	R Strain	S Strain	
1C+2A	4·74	1·93	Dicofol
1D i	2·65	0·83	Phenolic DDT
1D ii	2·15	0·49	DBP
2B	0·46	0·22	Phenolic DDT, phenolic DDE
2C	0·07	0·01	
2D	0·07	0·01	
3A i	0·85	0·03	Phenolic DDT, phenolic DDE
3A ii	0·59	0·30	
3B i	1·31	0·66	Carboxylic acid
3B ii	0·32	0·38	
3B iii	0·95	0·90	Phenolic DBP
3C	0·24	0·12	

* Neither dose lethal to strain of larvae concerned but dose given to R larvae lethal to S larvae.

† See Figure 1.

‡ See Figures 2 and 3, also Section III(*c*).

DDT hydroxylating and oxidizing systems were active in both resistant and susceptible larvae. Thus considerable quantities of DDT were converted to dicofol, DBP, and a phenol, and small quantities were converted to a carboxylic acid and to possibly other phenols. The last three mentioned types of compounds included six metabolites which could not be identified by the evidence available.

More of low doses was metabolized by susceptible larvae than by resistant larvae, but this could not have determined the relative susceptibility of the strains to DDT because the metabolites involved were considerably less toxic than DDT to susceptible larvae. Moreover, greater amounts of these metabolites were produced by resistant larvae surviving the highest dose than susceptible larvae surviving the lowest dose. In contrast to larvae, engorged female ticks produced no detectable quantities of these metabolites, yet in the resistant strain, both of these stages are equally resistant to DDT (Stone 1957).

It is clear from these observations that absorption or metabolism do not contribute causatively to specific resistance in ticks to DDT.

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