Colorimetric and Radiometric Determinations of DDT and Its Metabolites in Resistant Houseflies

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Studies on degradation products of DDT in seven DDT-resistant strains of houseflies, using radioactive DDT (carbon-14–labeled in the tertiary position), showed that the only significant product of DDT metabolism was DDE. Both DDT and DDE were found in the ether soluble portion of the excreta, the DDE-DDT ratio increasing with increasing time intervals. Very small amounts of a radioactive product were found in the water-soluble portion of the excreta. Losses of DDT were not consistent and are thought to be within the range of experimental error. No strain specificity was evident. In flies held 10 days after application of the insecticide, small but consistent losses of DDT were experienced, which might be attributed to incomplete recovery of material from excreta.

INVESTIGATIONS ON THE PHYSIOLOGICAL ASPECTS of DDT resistance in houseflies showed that resistant strains of houseflies are able to convert DDT into the relatively nontoxic derivative, 2,2-bis - (*p* - chlorophenyl) - 1,1 - dichloroethylene (DDE).

Several investigators (*1*, *10*, *14*, *15*) concluded that in addition to DDE another metabolite of DDT is produced which does not respond to the Schechter-Haller colorimetric method of analysis. This conclusion was based on the fact that in many instances the amounts of DDT and DDE recovered from houseflies exposed to DDT did not account for all the insecticide that was applied. Quantitative measurements of this unknown substance, designated in the literature as metabolite X or as non-Schechter-Haller compound and ranging from a few per cent to as much as 30 to 40%, were obtained by subtracting the observed quantities of DDT and DDE from the theoretical amount of DDT applied. Results of other investigations with resistant houseflies using the radioactive bromine (Br^{82}) analog of DDT (*17*) showed nearly quantitative recoveries as DBrDT and DBrDE, with no evidence of other metabolites. The formation of metabolites of DDT other than DDE by various other insects—e.g., the milkweed bug (*6*), the American roach (*2*, *3*, *16*), the Mexican bean beetle, the red-banded leaf roller, and two species of grasshoppers (*13*)—has also been reported. However, the nature of these unknown metabolites has remained obscure.

As the evidence for the presence of unknown metabolites in houseflies is based entirely on analytical differences, a close check on the method of application and extraction, and analysis by both colorimetric and radiometric methods, appeared desirable. Accordingly, the present work was undertaken in an effort to make a quantitative study of the products of DDT metabolism in resistant houseflies using radioactive DDT (*8*). Studies were made on seven resistant fly strains obtained from various laboratories.

Methods and Procedures

Benzene solutions of DDT were applied topically with a 0.25-ml. tuberculin syringe whose plunger was actuated by motion of the shaft of an improved ratchet-type apparatus. In a typical experiment, 20 to 50 adult female flies, 3 to 4 days old, were anesthetized with carbon dioxide and 11 to 13 γ of DDT in 1 μl. of benzene was applied to the thoracic region of individual flies. Following treatment, the flies were placed in 400-ml. beakers and provided with food and water.

The procedure used for studying the degradation of DDT was as follows: At 24-hour intervals for a period of 96 hours and in some tests for 10 days after application of the insecticide the flies were ground in a mortar with anhydrous sodium sulfate and extracted with ether in a Soxhlet apparatus for 24 hours. Following evaporation of the ether, the residue was taken up in carbon tetrachloride and passed through a sulfuric acid–Celite column (*5*) to remove extraneous materials. The beakers containing the excreta were rinsed with ether, followed by thorough cleaning with sodium hydroxide using a rubber policeman. The alkali portion was acidified with 6*N* sulfuric acid and re-extracted with several portions of ether. This procedure was followed to recover DDT or metabolites, including those acidic in nature, which might appear in the feces. The total beaker extract was then combined with the fly extract. DDT and DDE were determined in aliquots of the combined extracts by the Schechter-Haller method, and DDT was determined by radiometric assay. Resolution of DDT and DDE in a mixture was accomplished by analysis of a two-component color system (*10*).

Application of accurately known dosages of insecticides using mechanical microapplicators is very difficult. Preliminary experiments indicated that the most reliable method of estimating the dosage applied was to analyze treated flies immediately after application. Accordingly, for each test zero-hour determinations of dosage were made by macerating 20 to 50 treated flies, extracting in a Soxhlet apparatus with ether, and analyzing by both colorimetric and radioassay methods. Results were taken as true average dosage applied for the respective experiment.

Radioassay Because of the extensive use of carbon-14–labeled DDT in this laboratory, a routine assay method of suitable precision was established (*4*) and applied to the present study. It consists of the wet oxidation of DDT to carbon dioxide with subsequent precipitation as barium carbonate. The carbonate is prepared under conditions that will give a compact filter cake 2.92 sq. cm. in area. Inert carbon in the form of stearic acid is added to provide precipitates of "infinite thickness."

Table I. Reproducibility of Determinations of Radioactive DDT in Varying Quantities and Weights of Barium Carbonate Precipitate

Sample	Radioactive DDT, γ	Barium Carbonate Ppt., Mg.	Net C.p.M.[a]/γ DDT	C.p.M.[a]/γ Corr. to 70 Mg. of Ppt.
1	4	70	44.0	44.0
2	4	121	24.5	42.4
3	8	71	43.8	43.3
4	8	122	24.8	43.2
5	4	68	48.2	46.8
6	4	121	25.2	43.6
7	8	73	43.0	41.3
8	8	123	25.0	43.9
			Arithmetic mean	43.6
			Standard deviation	1.48
			Coefficient of variation	3.4

[a] Counts per minute.

The weighed precipitates are assayed in a Q-gas flow counter (Nuclear-Chicago, Model D-46) recording at least 6000 disintegrations to minimize counting errors. All measurements are corrected to a precipitate weight of 24 mg. per sq. cm. Precipitate weights of samples ranged from 24 to 35 mg. per sq. cm. According to Sacks (*12*) this represents an error not greater than 5%. Some typical calibration data for the radioassay method are presented in Table I. The method shows good reproducibility with a coefficient of variation of 3.4.

Schechter-Haller Method Calibration data for the Schechter-Haller method have been published by various workers on numerous occasions. In the writers' experience the method has a coefficient of variation of 5.5 for the quantities of DDT used in this study. The accuracy of resolving DDT and DDE in mixtures by the two-component color system has been amply covered in another publication (*7*).

Experimental Results

A summary of the results obtained with seven DDT-resistant strains of houseflies is presented in Table II. The data represent the averages of at least two and in most cases three individual experiments for each strain.

An analysis of the variation between the two methods of assay shows that differences with respect to time interval in all the strains (Table III) and with respect to individual strains at all time intervals (Table IV) are not significantly greater than the deviation from the mean within each method and between methods of assay.

It is seen from Table II that total recoveries of DDT and DDE 24 hours after treatment were, in most cases, greater than 90%. At longer intervals small losses were encountered occassionally, but these were not consistent and showed no specific trend of decreasing amounts, as would be expected if other unknown metabolites were being produced in significant quantities. The close agreement between the colorimetric and radiometric assays also suggests good recovery in terms of Schechter-Haller positive compounds. If significant amounts of unknown non-Schechter-Haller positive metabolites were produced, one would expect a consistent difference in favor of radioassay. The results in Table II also demonstrate that the small losses encountered were not characteristic of any particular strain, indicating no strain specificity with regard to production of unknown metabolites.

On the other hand there are differences between the strains in the rate of accumulation of DDE. This has been shown to be due primarily to differences in rates of absorption of DDT between strains (*9*, *11*).

Analyses of DDT and DDE in flies held for 10 days after treatment consistently showed some loss, averaging

Table II. Radioassay of DDT and Colorimetric Determination of DDT and DDE

(At indicated intervals following topical applications of DDT to seven resistant strains of houseflies)

		Recovered, γ/Fly					
		Colorimetric				Radioassay	
Fly Strain	Interval to Extraction, Hours	DDT	DDE[a]	Total as DDT	%	Total as DDT	%
Roberds	0	11.6	0	11.6	...	12.0	...
	24	5.0	6.6	11.6	100	11.9	99.0
	48	2.8	8.9	11.7	101	11.0	91.6
	72	2.6	9.8	12.4	107	11.3	94.2
	96	2.1	9.8	11.9	102	11.1	92.5
	10 days	1.4	8.5	9.9	85.3	9.8	82.0
DDT-45	0	12.0	0	12.0	...	12.4	...
	24	7.9	4.1	12.0	100	12.5	101
	48	5.6	6.1	11.7	97.5	12.2	98.4
	72	4.2	6.8	11.0	91.6	11.7	94.3
	96	3.9	7.6	11.5	95.8	12.2	98.4
	10 days	1.8	8.5	10.3	86.0	10.8	87.0
DMC-5	0	11.9	0	11.9	...	12.6	...
	24	9.8	1.6	11.4	95.8	12.7	101
	48	7.3	2.9	10.2	85.7	12.2	96.8
	72	5.8	4.8	10.6	89.1	12.2	96.8
	96	4.5	5.3	9.8	82.3	11.4	90.4
	10 days	...	...	...	...	...	...
Orlando	0	12.2	0	12.2	...	12.2	...
	24	9.6	1.7	11.3	92.6	11.0	90.0
	48	8.4	2.3	10.7	87.7	11.1	91.0
	72	7.8	3.6	11.4	93.4	10.8	88.5
	96	7.6	3.6	11.2	91.8	11.1	91.0
	10 days	2.9	8.0	10.9	89.3	10.5	86.0
Edgewood	0	11.4	0	11.4	...	12.0	...
	24	8.7	2.8	11.5	101	11.6	96.6
	48	7.2	3.8	11.0	96.4	11.6	96.6
	72	5.6	5.3	10.9	95.6	11.3	94.2
	96	4.4	6.0	10.4	91.2	11.2	93.3
	10 days	1.4	8.6	10.0	87.7	11.0	91.6
Bellflower	0	12.8	0	12.8	...	13.0	...
	24	9.2	2.7	11.9	93.0	11.1	85.4
	48	7.3	4.7	12.0	93.7	11.5	88.5
	72	6.0	5.7	11.7	91.4	12.5	96.1
	96	4.5	6.6	11.1	86.7	11.6	89.2
	10 days	1.5	9.0	10.5	82.0	11.8	90.7
Urbana	0	13.1	0	13.1	...	13.3	...
	24	10.3	2.1	12.4	94.6	11.8	88.7
	48	8.4	3.7	12.1	92.3	12.3	92.5
	72	6.3	5.2	11.5	87.8	12.8	96.2
	96	4.9	5.7	10.6	81.0	12.3	92.5
	10 days	1.5	9.8	11.3	86.2	11.6	87.2

[a] Values expressed as molecular equivalents of DDT.

Table III. Average Difference between Schechter-Haller and Radiometric Determinations for All Strains at Various Time Intervals

Time, Hours	0	24	48	72	96	10 days
γ per fly	+0.3	−0.07	+0.3	+0.4	+0.6	+0.4
% difference[a]	+2.5	−0.6	+2.7	+3.5	+5.5	+3.8

[a] Based on average Schechter-Haller determinations for all strains at each time interval. Plus values indicate radioassay greater than Schechter-Haller assay.

Table IV. Average Difference between Schechter-Haller and Radiometric Determinations for All Time Intervals with Respect to Individual Strains

Strain	Roberds	DDT-45	DMC-5	Orlando	Edgwd.	Bellfl.	Urbana
γ per fly	−0.33	+0.55	+1.24	+0.07	+0.58	+0.25	+0.51
% difference[a]	−2.9	+4.8	+10.7	+0.6	+5.0	+2.0	+4.2

[a] Based on average Schechter-Haller determinations at all time intervals for each strain. Plus values indicate radioassay greater than Schechter-Haller assay.

about 14% for six strains by both methods of assay. If it is assumed that the radioactivity should not be lost, these losses might be interpreted as being due to unextracted material. It is not unreasonable to assume that after several days the insecticide and metabolites may be tightly bound in tissues or may be conjugated in some form that makes extraction more difficult. On the other hand, excretion of other metabolites might also take place in larger quantities. As holding time is increased, it also becomes increasingly difficult to obtain quantitative recovery of feces and extraction of fecal material. Thus, at the 10-day interval, a substantial amount of the losses encountered could have been due to some of these factors. Some of the points discussed above are further illustrated in Tables V and VI.

The results in Table V were obtained by direct Schechter-Haller analysis without prior extraction. In most cases five treated flies were nitrated directly using 20 ml. of nitrating mixture and increasing the nitration time to 2 hours. In these tests the excreta were carefully collected separately, suspended in water, and then extracted with several portions of ether. The water phase was dried over phosphorus pentoxide in vacuo and the residue analyzed. The ether phase was evaporated and the residue analyzed in the same manner. The results obtained by these procedures show that almost complete recovery by colorimetric analysis was realized 24 to 96 hours after application. Both DDT and what seemed to be DDE were recovered in the ether-soluble portion of the excreta and the ratio of DDE to DDT increased with time (Table V). No positive identification was made of the red-colored reaction product in the ether-soluble fraction of the excreta.

In other tests the excreta from separate groups of flies, held for the same periods of time, were extracted with water and ether as above and subjected to radioassay (Table VI). The total radioactive material in the ether phase cannot be compared directly with the corresponding amount obtained by the Schechter-Haller method in Table V, as separate lots of excreta are involved. Nevertheless, the amounts found in the two separate experiments are of the same order of magnitude, and both show increasing amounts of material present in the excreta with increasing intervals. Because of the very small amounts of material involved, it was not possible to perform colorimetric analyses of the water phase except at the 96-hour test. However, radioactive material was detected in the water phase at all intervals involved (Table VI).

The results presented in Tables V and VI indicate that a substantial portion of the dosage applied is found in the excreta, increasing with time to as much as 17% in 96 hours. Most of the material in the excreta is ether-soluble, but a small amount of radioactive water-soluble material is present to the extent of 6.9% of the total radioactivity in the excreta. In terms of the dosage of DDT applied per fly, this represents only 1.15% after 96 hours.

From the resistance standpoint, the very small amounts of metabolites other than DDE produced after extended intervals do not appear to be vital to the fly's survival. For practical considerations the dehydrochlorination process still remains the important factor in the fly's defense against the lethal action of DDT. The interesting but complex phenomena of production of other metab-

Table VI. Radiometric Assays of Ether-Soluble and Water-Soluble Fractions of Excreta of Treated Flies (DDT-45 Strain) at Indicated Intervals Following Topical Applications of DDT

Interval to Extraction, Hours	Recovered, γ/Fly			Water-Soluble Material in Excreta, %	% of Applied Dose as Water-Soluble Compound[a]
	Ether-soluble fraction	Water-soluble fraction	Total		
24	0.65	0.035	0.685	5.1	0.35
48	1.09	0.079	1.169	6.7	0.79
72	1.38	0.095	1.475	6.5	0.95
96	1.55	0.115	1.665	6.9	1.15

[a] As flies were from treated batch used in test described in Table V, dosage applied is taken as 10 γ/fly.

Table V. Colorimetric Determination of DDT and Metabolities by "Direct Nitration" of Treated Flies (DDT-45 Strain) and Excreta at Indicated Intervals Following Topical Applications of DDT

(Micrograms per fly)

Interval to Extraction, Hours	In Flies			In Excreta						
				Ether Phase						
	DDT	DDE[a]	Total	DDT	DDE[a]	Total	Ratio DDE/DDT	Water phase	Total Recovery	% Recovery
0	10.0	...	10.0	...	...	...	...	...	10.0	...
24	5.65	3.32	8.97	0.43	0.37	0.80	0.86	0.0	9.77	97.7
48	3.15	5.30	8.45	0.63	0.60	1.23	0.95	...	9.68	96.8
72	1.50	6.50	8.00	0.74	0.78	1.52	1.05	...	9.52	95.2
96	0.94	7.08	8.02	0.80	0.90	1.70	1.13	0.12[b]	9.84	98.4

[a] DDE values expressed as molecular equivalents of DDT.
[b] Reaction product showed light pink color indicative of Schechter-Haller positive compound, but owing to small quantities present this could not be ascertained.

olites remain, essentially, academic in nature.

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PESTICIDE RESIDUES

Schradan Residues in Cotton and Cottonseed Products

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Radioactive phosphorus-32-schradan was sprayed on cotton at the rate of 1.0 pound per acre and after 41 days the extent of contamination of leaves, seeds, raw and refined oils, cake, cottonseed meal, and soapstock was evaluated. Schradan showed a surprising affinity for the oily seeds, and about 8 to 16 p.p.m. was present in the raw oil. Upon refining, this was decreased to 0.02 p.p.m. and the schradan was transferred to the soapstock. Ground cottonseed meal and cake contained 70 to 80 p.p.m. of radioactive phosphorus-32 calculated as schradan, but the very low chloroform–1N sodium hydroxide partition ratios indicated that this material was completely metabolized to acidic products. The experiment demonstrates the value of radiotracer studies in evaluating the behavior of systemic insecticides.

THE SYSTEMIC INSECTICIDE OCTAMETHYL PYROPHOSPHORAMIDE (OMPA or schradan) has proved effective during the past several seasons in controlling red spider mites and aphids attacking cotton, and the compound has been in semicommercial use for this purpose. The dosages employed have ranged from 0.5 to 1 pound (approximately 0.5 to 1 pint) of technical material per acre applied at 5 to 25 gallons per acre by air or ground equipment. As a consequence of the large scale utilization of cottonseed oil in the preparation of food products, such as butter substitutes and cooking oils, it became of interest to establish the possible levels of contamination of cottonseed, oil, and other products resulting from this use of schradan. The use of a radiotracer seemed ideal for this purpose because of the extreme sensitivity of detection, and the simplicity of assay as compared with other analytical methods (*2, 3*).

Materials and Methods

Radioactive schradan was prepared from 3.35 grams of $P^{32}OCl_3$ (purchased from Tracerlab, Inc., Boston, Mass.) with an initial activity of 10 millicuries, by the following reactions (*1*).

$$P^{32}OCl_3 + 2(CH_3)_2NH \xrightarrow{\text{ether}} (Me_2N)_2\overset{O}{\overset{\|}{P}}{}^{32}Cl$$

$$(Me_2N)_2\overset{O}{\overset{\|}{P}}{}^{32}Cl + (Me_2N)_2\overset{O}{\overset{\|}{P}}OC_2H_5 \xrightarrow{170°} (Me_2N)_2{}^{32}P(=O)-O-\overset{O}{\overset{\|}{P}}(NMe_2)_2 + C_2H_5Cl$$

The final product was distilled at 121° (<1 mm. of mercury) and 6.3 grams was obtained with a relative activity of 4.7 counts per second per microgram. This product was very pure and behaved as a homogeneous compound in paper chromatography upon ethylene glycol-impregnated paper (*4*). When partitioned between chloroform and 1*N* sodium hydroxide, a ratio of 27.8 to 1 was obtained at 30° C.

For use in the spray application on cotton, the phosphorus-32 isomer was diluted by the addition of 4 grams of schradan prepared by the same process and redistilled out of the reaction vessel so as to recover additional tagged product. The final tracer preparation had a relative activity of 3.6 counts per second per γ.

The radioactive schradan was applied to a heavy stand of Acala 4-42 variety cotton at the Bowlin Ranch in Indio, Calif., on September 2. The temperature was about 100° F. Nine grams of the P^{32} OMPA were dissolved