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Unique Effects of DDT and Other Chlorinated Hydrocarbons on the Metabolism of Formate and Proline in the Housefly*

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Insecticide-treated and control flies were injected with C^{14}-labeled biochemicals, and 3 hours later the soluble radiometabolites were extracted for identification and assay by paper chromatography and radiometric techniques. Of the injected compounds, DDT was found to interfere most with the metabolism of formate, glycine, and proline. Thus after injection of C^{14}-formate, more uric acid and allantoin and less proline were recovered as radiometabolites from flies treated with DDT and related chlorinated hydrocarbon insecticides than from flies untreated or treated with nontoxic analogs. However, insecticides of other types such as pyrethrum, organic phosphates, and phosphonates and a carbamate, interestingly, failed to show a significant effect on formate metabolism, providing additional evidence for a different mode of action.

Although introduced about 20 years ago, DDT[1] still has the largest share in the volume of insecticides used throughout the world, and little has been learned about how this compound and other chlorinated hydrocarbon insecticides produce their toxic effects. This lack of knowledge concerning the mechanism of action greatly hampers efforts to evaluate the toxicity of such agents toward higher animals, as well as studies of the nature of insect resistance.

The presence of DDT as a trace component of fat in normal humans in many countries has become a well-established fact, but not one which is generally viewed with alarm by toxicologists, since experiments involving the exposure of human volunteers to relatively large amounts of DDT have not revealed toxic effects (Hayes *et al.*, 1956, 1958). However, until more knowledge of the mode of action of these agents has been obtained, there will remain some degree of uncertainty regarding the effects of long-term exposure to such materials.

Numerous data show a high degree of correlation between structure and activity in the case of DDT and related compounds (Metcalf, 1955), but conclusive biochemical evidence showing any specific enzyme or metabolic pathway to be the site of action of such compounds has yet to be found (Metcalf, 1955; Perry, 1960a,b). Strangely, *in vivo* studies involving normal body constituents tagged with radiocarbon appear to have been largely neglected in this field, with the exception of a report by Winteringham (1958) that DDT resembled an organophosphate in its effect on C^{14}-acetate metabolism in the housefly. Tracer techniques and paper chromatographic methods applicable to pesticide research have been discussed (Winteringham, 1960).

The present report describes *in vivo* housefly experiments involving thirteen C^{14}-labeled amino acids, purines, and their precursors. DDT and other toxic chlorinated hydrocarbon insecticides, in contrast to nontoxic analogs and other classes of insecticides, were found to be unique in stimulation of radiopurine synthesis and radioproline degradation in flies injected with C^{14}-formate.

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[1] Abbreviations and insecticide names: AIC, aminoimidazole carboxamide; DDA, bis(*p*-chlorophenyl)acetic acid; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DMC, bis(*p*-chlorophenyl)methylcarbinol; *o,p'*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane. For abbreviations and names of other insecticides see Kenaga (1960).

TABLE I

CHROMATOGRAPHIC DATA FROM RADIOMETABOLITES

Radiometabolite	R_F Values in Various Solvents					
	Formic[a]	Ammonia[b]	Phenol[c]	*s,t*-Bu[d]	*s*-Bu[e]	Ester[f]
α-Alanine	0.59	0.26	0.61	0.25	0.15	0.37
Allantoin	0.27	0.17	0.54	0.41	0.31	0.29
Glutamic acid	0.46	0.16	0.38	0.28	0.10	0.28
Glutamine	0.30	0.22	0.58	0.21	0.12	0.16
Proline	0.61	0.35	0.89	0.34	0.20	0.43
Serine	0.35	0.25	0.35	0.24	0.13	0.19
Uric acid	0.20	0.14	0.44	0.30	0.17	0.12

[a] *t*-Butyl alcohol, formic acid, water (70:15:15). [b] *t*-Butyl alcohol, methyl ethyl ketone, water, ammonium hydroxide (40:30:20:10). [c] Lower phase from phenol and water. [d] Upper phase from *t*-butyl alcohol, *s*-butyl alcohol, water (40:200:225). [e] Upper phase from *s*-butyl alcohol and water. [f] Ethyl acetate, formic acid, water (70:20:10).

METHODS

Topical Insecticide Treatments.—Three-day-old adult female flies of the DDT-susceptible NAIDM strain were placed in beakers and supplied with water and sugar cubes for 2 days prior to insecticidal treatment. The insecticide in benzene solution (DDA was dissolved in acetone) was applied by means of a micrometer-driven syringe to the abdomens of flies under carbon dioxide anesthesia. Each group of eight treated or untreated flies was then confined in a beaker without food or water for the next 3 hours preceding the injection of the isotopic compound.

The volume of benzene solution commonly used was 0.2–1.0 μl per fly. The nontoxicity of this solvent is evident from the data of Table II, which do not indicate any metabolic effects of benzene used alone (1–2 μl per fly) or as solvent (1 μl per fly) for compounds such as DDE, α-lindane, Kelthane, or DMC.

Injection of C^{14}-Biochemicals.—All radioactive compounds were purchased from Calbiochem[1] with the exception of 3-C^{14}-pyruvate, proline-5-C^{14}, and 8-C^{14}-adenine, which were obtained from New England Nuclear Corporation. In most cases the fly was injected with 1 μl of aqueous C^{14}-sodium formate (0.05 μc, 0.28 μg, 11.8 mc/mmole). Isotopic compounds were usually dissolved in water, but in a few early experiments cockroach saline (Yeager, 1939) was employed. The solution was injected into the side of the thorax of the carbon dioxide–immobilized fly by means of a micrometer-driven 50-μl Hamilton syringe (#705), viewed under a low-power microscope. Each group of eight injected flies was then confined in a 10-ml vial or a 7-ml tube of a tissue grinder (Corning Glass #7725) with the open end in each case closed off by a wire screen.

Extraction of Radiometabolites.—Three hours after injection of the C^{14}-biochemical the flies were killed by 1 ml of absolute ethanol poured through the wire screen into the container. The screen was removed and homogenization performed immediately. In vials, the grinding was accomplished with the aid of sand and a glass rod flattened on one end, whereas in tissue grinders pestles were used. One ml of 80% ethanol was then added (in a few cases 0.2 ml of water was added instead) and the tube manipulated so as to wash down all fly excretion material. After centrifugation of the vial or tube, an aliquot (usually 0.2 ml) of the supernatant was used for paper chromatography. Insecticide dosages were limited so that all flies survived until homogenized.

[1] Use of trade names and the names of suppliers is for identification only, and does not constitute product-endorsement by the Public Health Service.

Paper Chromatography.—Ascending two-dimensional chromatography employing 6-cm square sheets of Whatman 1 filter paper was conducted in small rectangular Thomas-Kolb jars (A. H. Thomas Co.) at 24–26°. Radioactive compounds were located on the chromatograms by the use of X-ray film (5 × 7 in.). To provide data for identification (see Table I) the radiometabolites were eluted by means of a special apparatus from the primary chromatogram and rechromatographed. To elute and transfer to a new sheet of paper, a chromatographic spot was cut out with a point on one end and mounted with the point upward and projecting beyond the rim of a small tube which held enough 80% alcohol to immerse the bottom of the cutout. The tube, equipped with a float, was raised and lowered in a water bath by an automatic siphon so that the cutout point intermittently touched the new paper at a desired point. Some radiometabolite was transferred by capillarity at each contact and the solvent evaporated during each interval so that the size of the resultant spot could be controlled.

The identifications of all radiometabolites of Table I, except uric acid, were verified by cochromatography with nonradioactive known compounds in all six solvent systems. A drop of solution of nonradioactive known compound was applied to the cutout paper being eluted in the special device; and after development of the new chromatogram, the outline of the radiometabolite on the autoradiogram was found to match the outline of the authentic compound on the sprayed chromatogram. Ninhydrin was used as spray reagent for amino acids and Ehrlich's reagent for allantoin. A recent publication contains R_F values for a large number of known compounds in solvents b–f and in a "Form, *t*-Bu" solvent which gives values very similar to our solvent a (Fink *et al.*, 1963).

Quantitative Radioassay of Metabolites.—To obtain the radioactivity data in Tables II and III, each aliquot of supernatant of fly extract was chromatographed two-dimensionally in solvents a and b successively. Radiometabolites located by autoradiography were then encircled on the chromatogram, uric acid and allantoin being taken together. Each outlined area was then cut out and dropped into 1 ml of water in a scintillation vial. One or more hours later, 20 ml of scintillation fluid was added to each vial. The fluid had the composition recommended by Kinard (1957) with the exception of toluene in place of xylene. The radioactivity was then determined in a liquid scintillation counter (Tracerlab, Inc.). The activity of 1 μl of radioformate (0.05 μc), assayed under the same conditions, was 64,500 cpm.

RESULTS

Effects of Insecticide on Formate Metabolism.—Figure 1 presents autoradiograms of chromatograms prepared

TABLE II

EFFECTS OF VARIOUS TREATMENTS ON RADIOMETABOLITE CONTENT OF C^{14}-FORMATE-INJECTED FLIES[a]

Expt.	Topical Treatment: Compound	Topical Treatment: Dose/fly (μg)[b]	% Knockdown	Radioactivity of Metabolites (cpm/fly): Uric Acid, Allantoin	Proline	Serine
1[c]	None		0	2100	1820	2820
	DDE	20	0	1010	1870	2250
	DDT	1	100	8760	810	1910
	Dieldrin	2	100	5770	980	1740
2[c]	None		0	750	2370	3110
	Lindane	0.3	100	3880	1060	3330
	α-Lindane	20	0	1200	2330	2520
	DDA	10	0	680	3220	2370
	Kelthane	10	38	870	3370	3610
3	None		0	930	2000	990
	Pyrethrum	0.8	100	1070	1660	2410
	DDD	5	100	4070	480	1750
	DDT	0.4	100	3130	1020	1090
4	None		0	1290	1330	1190
	Dipterex	0.6	100	1150	2350	1370
5	None		0	1230	2140	1110
	DMC	10	0	1160	2940	890
6	None		0	1300	1690	810
	Prolan	0.6	88	5300	170	620
	DDT	0.2	75	7280	220	470
7	None		0	1550	4920	3760
	Methoxychlor	0.5	100	6300	1640	3460
	Heptachlor	0.5	100	5750	3060	2540
	Malathion	0.8	38	2760	4180	2610
	DDT	0.2	88	4530	2010	3440
8	None		0	2800	3300	1180
	TEPP	0.24	63	2360	3200	2520
	para-Oxon	0.038	100	960	2990	5560
	Parathion	0.025	100	2140	2800	3170
9	None		0	1200	2270	1660
	Sevin	15	50	1100	2800	3170
	Toxaphene	3	88	5830	880	1500
10	None		0	2390	2820	1410
	Benzene	1 μl	0	2850	3340	1920
	Benzene	2 μl	0	2780	3370	1810

[a] For details of treatment and assay see Methods. [b] Volume of solution applied was 0.2–1.0 μl in experiments 1–7 and 1–3 μl in experiments 8 and 9. [c] Radioformate dissolved in saline After homogenization 0.2 ml of water was added.

TABLE III

VARIATION OF RADIOMETABOLITE CONTENT OF C^{14}-FORMATE-INJECTED FLIES WITH INSECTICIDE[a]

Expt.	Topical Treatment: Insecticide	Topical Treatment: Dose/fly (μl)	Topical Treatment: Dose/fly (μg)	% Knockdown	Radioactivity of Metabolites (cpm/fly): Uric Acid, Allantoin	Proline	Serine
1	None			0	1230	2140	1110
	p,p'-DDT	0.5	0.1	50	3150	920	840
	p,p'-DDT	1.0	0.2	88	7290	350	640
2	None			0	830	1560	1480
	o,p'-DDT	0.5	5	13	1680	770	1110
	o,p'-DDT	1.0	10	75	2690	340	1510

[a] For details of treatment and assay see Methods.

from extracts of DDT-treated and control flies injected with radioformate. The injections were performed 3 hours after treatment and 3 hours before homogenization-extraction. These results are from male flies but similar results were obtained from females. The identified metabolites are proline, serine, glutamic acid, uric acid, and allantoin. Spot A_2 is allantoin and spot A_1 is a compound which is rather unstable, giving rise to allantoin plus traces of urea and an unknown substance during elution and rechromatography. Since such procedures applied to authentic C^{14}-uric acid provided the same degradation products, it appears that A_1 is uric acid and in further discussion this is assumed. The same formate metabolites, with the exception of allantoin, have been identified in roaches by McEnroe and Forgash (1958). Uricase, the enzyme which degrades uric acid to allantoin, has been demonstrated in blowflies (Brown, 1938).

It is evident from Figure 1 that DDT treatment markedly affected the concentrations of three radio-

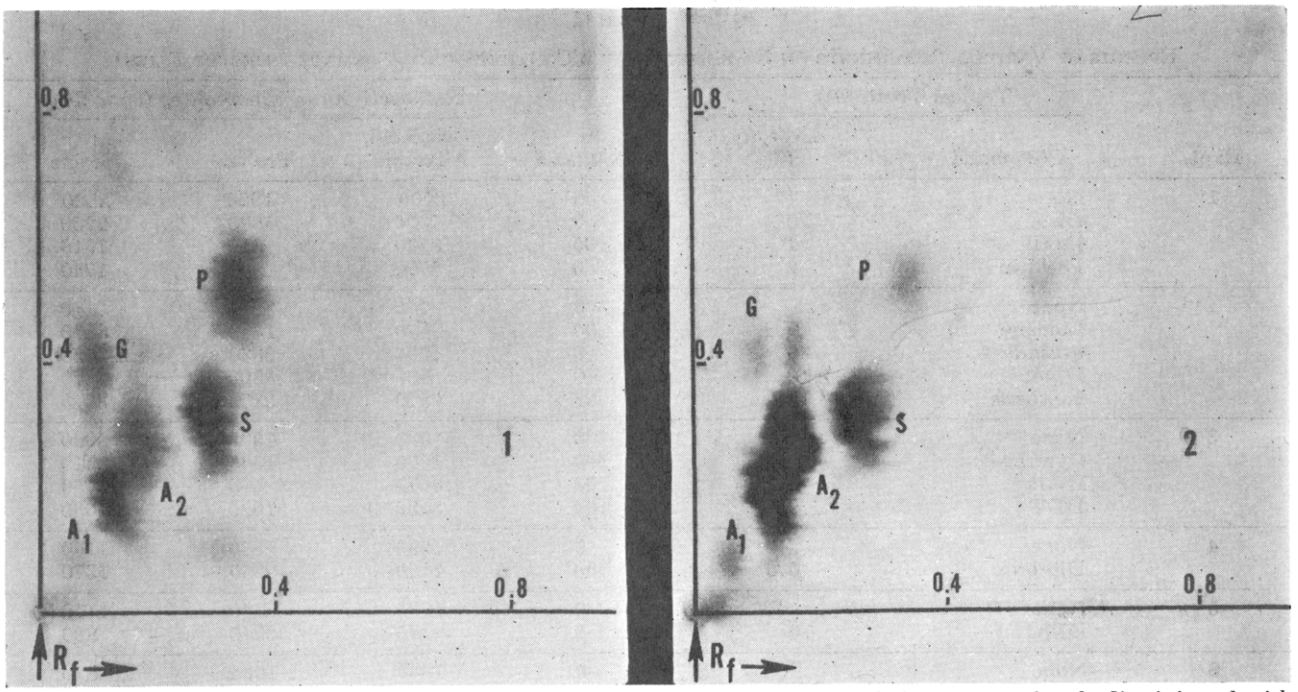

FIG. 1.—Autoradiograms of two-dimensional chromatograms prepared from alcoholic extracts of male flies injected with C^{14}-formate: (1) untreated; (2) treated with DDT. Radiometabolites: A_1, uric acid; A_2, allantoin; G, glutamic acid; P, proline; S, serine. Ascending chromatography first in solvent a (vertical direction in Figure 1) and second in solvent b (horizontal direction).

metabolites, causing a reduction in the content of radioproline and increases in the levels of radioactive uric acid and allantoin. On the other hand, DDT had little effect on the content of radioserine. Differences in spot intensities of minor constituents such as glutamic acid and unknown compounds may be worth further investigation.

The effects of a number of insecticides and related compounds on formate metabolism are presented in Tables II and III. Insecticides giving results similar to DDT were all the toxic chlorinated hydrocarbons tested, namely, dieldrin, lindane, DDD, Prolan (a nitro compound related to DDT), toxaphene, methoxychlor, heptachlor, and *o,p'*-DDT. The data show that for a given metabolic response the less toxic the analog the greater the dose needed. Thus roughly ten times as much DDD was required to cause the per cent knockdown and metabolic response attained with a given dose of DDT; the minimal dosage required for 100% knockdown was found to be 3–4 μg in the case of DDD and 0.3–0.4 μg for DDT, on the basis of results some of which are not included in Table II. None of the nontoxic analogs of the chlorinated hydrocarbons appreciably altered formate metabolism, as, for example, DDE, α-lindane, DDA, Kelthane, and DMC.

Other types of insecticides, not falling in the chlorinated hydrocarbon category, were not found to influence formate metabolism; for example, the carbamate Sevin, pyrethrum, the organophosphonate Dipterex, the organic phosphates TEPP and *para*-oxon, and the organic thiophosphates malathion and parathion. The data indicate that very few insecticides had a marked effect on the content of radioserine; the effect was largest in the case of *para*-oxon and also noteworthy in the case of Sevin and pyrethrum.

Dose-Response Correlation.—The data of Table III indicate for each of the two DDT isomers correlations between dosage, per cent knockdown, and metabolic effects. Thus the greater the dosage of *p,p'*-DDT used for treatment, the higher the per cent knockdown, the more isotope incorporated into uric acid and allantoin, and the less the content of radioproline. A similar correlation is indicated with the much less toxic *o,p'*-DDT, but the metabolic effects for this isomer are of a lower order of magnitude.

Distribution of Injected Activity.—After the flies were injected with C^{14}-formate, up to 92% of the activity was accounted for in the total of three fractions, namely, expired carbon dioxide, ethanolic extract, and residue. Radioactive carbon dioxide expired by the flies or evolved by combustion (Coleman Carbon-Hydrogen Analyzer, Model 33) of the fly residue was trapped in a solution containing ethanolamine (Jeffay and Alvarez, 1961) for scintillation assay. In a typical experiment involving twenty flies of the NAIDM strain in each group, the controls expired 49% of the activity during the 3-hour metabolic period following injection, whereas those treated each with 0.3 μg of DDT 3 hours before injection expired only 37% of the activity during the metabolic period. However, at the end of the metabolic interval the treated flies yielded more activity (38%) in the 10-ml alcoholic extract (80% ethanol) than did the control flies (25%). Combustion of the residue remaining after extraction, in both groups, led to recovery of 17% of the activity in the carbon dioxide trap. Total recoveries were 91% and 92% for the control and treated groups, respectively. These data, indicating a diversion of formate metabolism from respiratory channels to purine synthesis during the prostrate phase of DDT-poisoning, appear compatible with results reported by Fullmer and Hoskins (1951), who demonstrated fly respiration to be subnormal during the prostrate phase of poisoning, in contrast to the increased respiration during the earlier hyperactive phase.

The distribution of radiometabolites between the head, thorax, abdomen, and excretions was determined 3 hours after injection of radioformate into female flies of the CSMA strain (DDT-susceptible), treated each with 0.3 μg of DDT. In the head and thorax most of the activity was found in proline and serine,

TABLE IV

RADIOACTIVE COMPOUNDS EXTRACTED FROM FLIES AFTER INJECTION WITH VARIOUS C^{14}-BIOCHEMICALS[a]

Compound Injected (mc/mmole)	Injected/Fly (μc)	Injected/Fly (μl)	Extractants Detected on Chromatograms: Major	Extractants Detected on Chromatograms: Minor
Adenine-8-C^{14} (2.5)	0.05	1.0	Adenine, uric acid allantoin, one unknown	
AIC-2-C^{14} (2.9)	0.033	1.0	Uric acid, allantoin, AIC	
Betaine methyl-C^{14} (0.7)[b]	0.025	1.0	Betaine	
Glycine-2-C^{14} (5.0)[b]	0.025	1.0	Uric acid, allantoin	Proline, serine
DL-Proline-carboxyl-C^{14} (2.7)[b]	0.013	0.5	Proline	
DL-Serine-3-C^{14} (2.9)[c]	0.075	1.5	Serine	Proline, allantoin
Sodium acetate-1,2-C^{14} (10.5)[c]	0.038	1.5	Glutamine, proline	Glutamic acid
Sodium pyruvate-3-C^{14} (2.0)[b]	0.025	1.0	Proline, 2 unknowns	α-Alanine, allantoin
Succinic acid-2,3-C^{14} (13.7)[b]	0.013	0.5	Proline	
Uric acid-2-C^{14} (1.0)	0.005	2.0	Uric acid	
L-Glutamine-U-C^{14} (2.3)	0.025	1.0	Glutamine, proline	Glutamic acid
DL-Proline-5-C^{14} (6.9)	0.05	1.0	Proline	Glutamic acid, glutamine

[a] See Methods for experimental details. [b] Dissolved in saline. [c] Homogenized 2 hours after injection.

whereas in the abdomen and in excretions nearly all the activity was recovered in the uric acid and allantoin.

Time Factors.—At different intervals after injection of untreated flies with radioformate the contents of soluble radiometabolites were estimated on chromatograms. One hour after injection proline and serine accounted for most of the activity. By 3 hours after injection the radioserine content was largely depleted, the radioproline level slightly reduced, and the amount of radiopurines increased. When the flies were kept under carbon dioxide anesthesia for 3 hours after C^{14}-formate injection the labeled pool was found to consist largely of serine and a lesser amount of proline, on the basis of tentative identifications. A similarity between the effects of anoxia and *para*-oxon (see Table II) on formate metabolism is to be noted.

Different stages of DDT-poisoning are apparent in results from a study of radiopurine production as a function of time. Thus when the interval between treatment with 3 μg of DDT and injection of 1 μl of radioformate per fly was increased from 0.5 to 2 hours, the radiopurine production determined 1 hour after injection increased about 2-fold. At longer intervals, there was very little further increase.

Adjuvant Substrate Effects.—The incorporation of label into purines in untreated flies was enhanced about 12-fold by addition of 0.1 μmole of nonradioactive glutamine to the radioformate solution injected. Other unlabeled adjuvants such as α-alanine, glutamic acid, glycine, histamine, proline, serine, and tryptophane, used at the same molar concentration as glutamine, did not significantly affect the pattern of labeling. The extracts were made 3 hours after injection.

Nonradioactive Experiment.—Fly extracts were assayed for allantoin by adapting a column chromatographic procedure applied by Crokaert (1959) to urine. Each group of 85 flies was homogenized-extracted with 80% ethanol (portionwise). The combination of extracts totaling 50 ml was then concentrated and purified by passage through a Dowex-50 column (7 × 1.9 cm). After fractionation on another column (30 × 1 cm) of the same resin, each tube (1.2 ml) of eluate was assayed colorimetrically, both by the Koritz-Cohen method and by paper chromatography with the use of Ehrlich's reagent. Treated flies were found to contain about 50% more allantoin 6.5 hours after exposure to DDT (0.3 μg per fly) than did the control flies.

Metabolism of Other C^{14}-Biochemicals and DDT Effects.—The first column of Table IV lists the C^{14}-labeled biochemicals which were injected into control and DDT-treated flies. In other columns are listed radioactive compounds detected in alcoholic extracts of the flies 2–3 hours after injection. Depending on the precursor injected, the only labeled extract constituents for which levels have been found to be altered by exposure of the flies to DDT are uric acid, allantoin, and proline. Thus DDT stimulation of radiopurine synthesis was marked when glycine-2-C^{14} was injected but not when the purines, AIC-2-C^{14}, adenine-8-C^{14}, or 2-C^{14}-uric acid were injected. However, in all experiments in which proline was a labeled pool constituent, its degradation was found to be accelerated in DDT-treated flies. The identification of proline, glutamine, and glutamic acid as metabolites of acetate is in agreement with the isotope studies in flies reported by Price (1961).

DISCUSSION

The metabolic studies involving the injection into houseflies of a variety of C^{14}-labeled biochemicals have revealed three radiometabolites, namely, uric acid, allantoin, and proline, the production of which are markedly influenced by DDT-poisoning. Thus DDT stimulated the incorporation of label from injected C^{14}-formate or 2-C^{14}-glycine into the purines while it depressed the level of radioproline.

In experiments involving a variety of insecticides and related compounds, alterations in formate metabolism similar to those elicited by DDT appeared to be restricted to the chlorinated hydrocarbons, and the more toxic the chlorinated hydrocarbon or the higher the dosage, the greater the metabolic response, with nontoxic analogs such as DDE and α-lindane causing no response at all. Most significantly such changes in formate metabolism were not appreciable after toxic doses of other classes of insecticides such as pyrethrum, the carbamate Sevin, and phosphorus-containing compounds. These metabolic experiments provide evidence that the chlorinated hydrocarbons operate by a different mechanism from that of the other types of insecticides.

It seems most reasonable to assume that the effect of DDT on formate and glycine metabolism is secondary to a more primary effect involving stimulation of the breakdown of certain nitrogen compounds, with glutamine ⟷ glutamic acid serving as an intermediate carrier of nitrogen. Although little is known about the pathway of purine synthesis in insects (Gilmour, 1961), the data presented in this paper showing the utilization

of formate, glycine, AIC, adenine, and uric acid for allantoin synthesis are consistent with the assumption that the vertebrate pathway applies to insects. Of these precursors DDT stimulated the incorporation of label only from C^{14}-formate and glycine-2-C^{14} into uric acid and allantoin. By analogy with higher organisms, such an effect could be brought about through an increased rate of synthesis and breakdown of glutamine, since formate, glycine, and glutamine constitute three important starting materials in the purine synthetic pathway. Indeed, the synthesis of radioactive purines in untreated flies was greatly enhanced by injecting nonradioactive glutamine along with the radioformate. Injection of glutamine-U-C^{14} did not lead to recovery of a significant amount of labeled purines even from flies poisoned with DDT. Therefore it may be assumed that the glutamine nitrogen but not glutamine carbon can play an important role in these insecticidal effects.

Results from isotopic experiments indicate that, even after poisoning with DDT, proline does not contribute a significant amount of carbon for purine synthesis in flies, although proline may be presumed to be an important source of nitrogen. Proline and glutamine appear to be similarly interconverted in flies as in higher animals. Thus after injection of glutamine-U-C^{14}, proline was the major radiometabolite found in the fly extract, and after injection of proline-5-C^{14} traces of labeled glutamine and glutamic acid were detected in the extract.

The data indicating a selective depletion of proline in contrast to serine in flies exposed to toxic chlorinated hydrocarbons may lend significance to the excessive neuromuscular activity caused by such toxins, in that the proline metabolite, γ-aminobutyric acid, was thought to be uniquely found in the brain of higher organisms, where its level is related to seizures (Baxter and Roberts, 1960). Recently this amino acid has also been detected in the housefly, and its exclusive location in the head suggests a role in nerve metabolism (Price, 1961). A depletion of proline in roaches after exposure to DDT has been reported (Corrigan and Kearns, 1958). Another interesting metabolic effect concerns α-alanine, which had a higher concentration in resistant than in susceptible mosquito larvae after treatment with DDT (Micks *et al.*, 1960).

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