Metabolic Fate of DDT in *Drosophila melanogaster*. **II. DDT-Resistance and Kelthane-Production.** Masuhisa TSUKAMOTO (Genetical Laboratory, Faculty of Science, Osaka University, Osaka). Received Oct. 25, 1960. *Botyu-Kagaku*, **25**, 156, 1960. (in English)

28. ショウジョウバエにおける DDT の代謝. II. DDT 抵抗性と代謝.

塚本増久 (大阪大学理学部 生物学教室) 35. 10. 25 受理

第1報において DDT 抵抗性のキイロショウジョウバエは DDT をそのエタノール型の誘導体である Kelthane に代謝することを定性的に証明したが，この代謝経路は今迄全く知られていなかったので，その定量的な資料を提出するためと，それによって DDT 抵抗性の程度や抵抗性遺伝子と DDT の代謝との関係を知るために，体内で作られた Kelthane の量を Fujiwara 法で比色測定した．その結果，抵抗性系統や，抵抗性系統と非抵抗性系統との雑種では，DDT 処理後多量の Kelthane が作られることや，非抵抗性系統にも僅かながら DDT を代謝する能力があることなどが明らかとなったが，代謝のさかんなことが，抵抗性の強い原因であるのか，または代謝がはたして第2染色体上の抵抗性遺伝子によって支配されているのかどうかなどについては断定を下すことはできなかった．

Quantitative analyses for Kelthane production *in vivo* were colorimetrically carried out by the Fujiwara reaction from a genetical standpoint after rearing larvae of *Drosophila melanogaster* on media containing various amounts of DDT. DDT was metabolized not only by resistant strains but also by non-resistant strains, and the amounts of Kelthane produced were not directly proportional to the resistance levels of the strains used. It is not yet conclusive whether the metabolism is a cause of the resistance or a consequence of the resistance and whether the metabolism is due to the DDT-resistant gene.

Several years ago it was reported that DDT-resistance in *Drosophila melanogaster* was due to a dominant gene located at a particular part of the second chromosome[5,6]. In the first paper[6] of this series, it has been shown qualitatively that DDT-resistant strains of this insect species are capable of metabolizing DDT to its ethanolic derivative, Kelthane, but not to ethylenic DDE. These results suggested that the resistant gene in *Drosophila*, unlike that in the housefly, did not give rise to DDT-dehydrochlorinase activity.

The purpose of this paper is to present quantitative data on the DDT-Kelthane conversion and to ascertain the relationship between DDT-resistance and DDT-metabolism from a genetical standpoint. For this purpose, the production of the metabolite Kelthane *in vivo* was colorimetrically determined by the Fujiwara reaction after rearing *Drosophila* on media containing various amounts of DDT.

Materials and Methods

Various resistant and non-resistant strains of *Drosophila melanogaster* were used in this investigation. The following is a list of these strains with brief descriptions :

(1) *Canton-S*……highly susceptilble to DDT, BHC, parathion, nicotine sulfate, etc. Morphologically wild-type laboratory strain.

(2) *cn bw*……a second chromosomal mutant strain marked with two eye-color genes : *cinnabar* and *brown*. Susceptible to DDT.

(3) *bw* ; *st* ; *sv*^{n}……a multichromosomal mutant strain marked with three visible characters : *brown* eyes, *scarlet* eyes and *shaven-naked* bristles on the second, third and fourth chromosomes, respectively. Less susceptible to DDT than the usual mutant strains but susceptible to nicotine sulfate.

(4) *Hikone-R*……a multiple resistant strain to DDT, BHC, parathion, nicotine sulfate, etc. Collected as a DDT-resistant strain from the field in 1952. Subsequently selected under laboratory pressure by various insecticides for several years. Phenotypically wild-type.

(5) *WMB*……selected for DDT-resistance from

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a wild-type mixed population, *WMA*, inintially by Mr. T. Hiroyoshi and subsequently by the author for 4 years (more than 130 generations). Highly resistant to DDT. Wild-type.

(6) *WMD*……selected for Dipterex-resistance from the WMA strain for more than 100 generations. Resistant to DDT and Dipterex. Wild-type.

(7) *WMH*……selected for gamma-BHC-resistance from the WMA strain for more than 80 generations. Resistant to BHC and DDT. Wild-type.

(8) *TDE-R*……selected for TDE-resistance for more than 35 generations from a mixed population of the WMB, WMD and WMH strains. Highly resistant to DDT and TDE. Wild-type.

(9) *KSL*……obtained from Dr. B. Rasmuson, Sweden, as an organophosphate-resistant strain in 1958. Since then no selection with insecticides has been attempted in this laboratory.

For the physiological and biochemical investigations, special synthesized strains were also made up from the susceptible Canton-S strain and the resistant Hikone-R strain by the chromosome-substitution technique.

(10) *HR-1*……The X-chromosome of the Canton-S strain is replaced by that of the resistant strain, but the second and third chromosomes are derived from the susceptible strain. Susceptible to DDT.

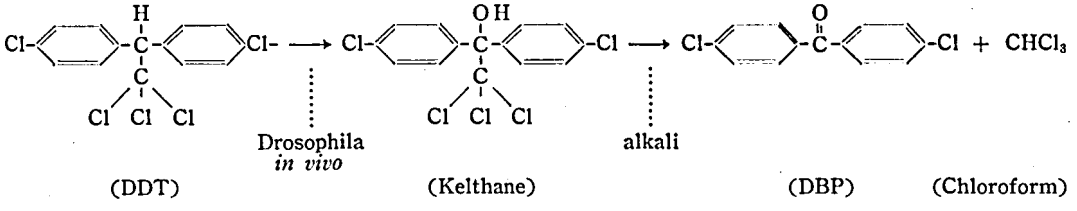
(11) *HR-2*……The second chromosome of the susceptible strain is replaced by the resistant second chromosome of the Hikone-R strain. Resistant to DDT.

(12) *HR-3*……The third chromosome of the susceptible strain is replaced by that of the Hikone-R strain. Non-sesistant to DDT but resistant to nicotine sulfate.

The insecticides used as selecting agents for increasing or testing resistance levels of various strains were furnished respectively by the following chemical companies: DDT, Asahi Glass Co. : TDE, Rohm & Haas Co. ; BHC, Asahi Glass Co. ; Dipterex, Nihon Tokushu Noyaku Co. ; Diazinon, Chugai Seiyaku Co. ; and nicotine sulfate, Japan Agr. Insecticide Co.

The techniques for insect rearing procedure, treatments with insecticides, tests for resistance and extraction of the metabolite were almost identical to those described in previous papers[6,8].

Analysis by the Fujiwara reaction. The metabolite Kelthane is degraded by alkali into dichlorobenzophenone (DBP) and chloroform as follows: the chloroform can be detected as a pinkish color complex by the Fujiwara reaction. Prior to colorimetric analysis, it is necessary to perform some preliminary purification of the ether extract of the insect sample because this contains large amounts of fatty substances which interfere with the analysis. The use of concentrated H_2SO_4 with cyclohexane or in the modified Davidow column[6] is highly effective for removing these interfering substances from the sample, but the use of the concentrated H_2SO_4 is not possible with the Fujiwara reaction because of the poor and/or variable recovery of Kelthane. To remove most of the interfering substance, therefore, the partition method between petroleum ether and acetonitrile



suggested by Hoskins *et al.*[1] has been employed prior to the Fujiwara reaction, and this is the most important difference in technique from that described in the part I of this series. The residue of ether extract from each gram of insect material was dissolved in a total of 30 ml. of petroleum ether and was transferred into a 100 ml. separatory funnel. Then 30 ml. of acetonitrile was added and the mixture was shaken for two minutes. After the two phases had completely separated, the lower acetonitrile layer was collected into a flask, the solvent was evaporated and the residue was transferred with a small portion of ether into a test tube. The ether was evaporated to dryness and then the residue was redissolved in 8.0 ml. of

pyridine, and test tube was chilled by ice-cold water. Just before the analysis, 3.0 ml. of chilled NaOH solution (15g. NaOH and 30 ml. H_2O) was added into the tube, and the tube was immediately shaken in a boiling water bath. After development of full color, which usually took 2-3 minutes, the upper pyridine layer was transferred into a glass cell with a 1-cm. light path, and the absorbance was recorded by a spectrophotometer. The amount of Kelthane was estimated from the difference between the absorbance at 538 mμ and that at 700 mμ. Distilled water was used as the reference solution. Standard concentration-absorption calibration curves were obtained from 1-gram samples of insect materials to which known concentrations of Kelthane had been added.

Experiments and Results

A. Kelthane Formation during Developmental Stages

When *Drosophila* larvae were reared on DDT-containing media, Kelthane was found not only in the larvae, but also in the pupae and resulting adult flies. Moreover, when adult flies reared on DDT-free media were placed in contact with DDT deposits, Kelthane was also detected even within 5 hours after the treatment.

Table 1 represents the Kelthane content found during the metamorphosis when first-instar larvae of the Hikone-R strain were bred on media containing DDT at 1000 μg/ml. Full-grown larvae were collected just before pupation, and middle stage pupae were collected by flotation on water; adult flies were collected within 24 hours of emergence. Larvae and pupae were washed in water, rinsed twice with ethanol and then twice with ether in a beaker. Abult flies were rinsed directly twice with ether. The insects were then spread on a dry filter paper, the ether instantly evaporated, and the material was immediately weighed out in 1-gram samples.

As shown in this table, the variance in Kelthane content is the least in the pupae. The pupae is a closed system without any uptake of food or excretion of feces. Although more troublesome to collect, pupal samples were employed for analytical materials in subsequent experiments.

Table 1. Kelthane content of the various stages in the Hikone-R strain exposed as larvae to 1000 μg DDT per ml. medium. Averages with fiducial limits at 5% level.

Developmental stage	No. of samples	Kelthane content μg/g	Kelthane content μg/individual
Larvae	6	107±31	0.097±0.033
Pupae	8	246±24	0.233±0.018
Adults	5	377±56	0.258±0.041

B. Effect of DDT Dosage on Amounts of the Metabolite

The Hikone-R strain was bred on media containing DDT at various dosage levels and each 1-gram pupal sample was analysed. Table 2 summarizes the results in micrograms Kelthane per gram pupae.

Table 2. Amounts of Kelthane produced in pupae of the Hikone-R strain at various doses of DDT.

DDT dose in μg/ml	No. of samples	Kelthane content in μg/g	Class
30	6	34±18	low
50	8	41± 7	
100	6	116±31	medium
200	8	119±12	
500	6	131± 8	
1000	8	257±35	high

In this table, the data can be grouped into three classes in respect of Kelthane content: low, medium and high. Differences among these three groups are highly significant, whilst there is no significant difference within a group at the 5% level.

C. Comparison of Kelthane Content among Resistant Strains

Levels of resistance to DDT, BHC and Dipterex in several resistant strains are compared in Table 3. All these strains are highly resistant to DDT, whilst the susceptible Canton-S strain cannot emerge normally even at 50 μg/ml dose of DDT. All the resistant strains are similar in their response to DDT, but differ in their response to BHC or Dipterex especially at the higher doses.

Larvae of certain of the resistant strains were reared on media containing DDT at 200 and 1000 μg/ml, and the content of the metabolite was

Table 3. Resistance patterns of five DDT-resistant strains to insecticides.

Insecticide	Dose in μg/ml	Percent emergence				
		Hikone-R	WMB	WMD	WMH	KSL
DDT	1000	75.4	95.6	82.6	89.2	89.0
	2000	85.6	94.2	75.4	87.8	84.6
	3000	85.0	94.6	74.8	88.8	81.0
	4000	78.2	90.6	72.2	88.2	83.2
BHC	10	68.8	94.0	73.4	82.2	92.8
	20	51.6	53.2	68.0	69.2	3.0
	30	13.4	43.6	46.0	54.6	0.0
Dipterex	1.0	90.3	91.2	88.8	71.4	89.2
	3.0	37.6	95.6	88.4	3.6	78.0
	5.0	14.4	55.8	89.8	0.2	0.0

quantitatively analysed in the pupae (Table 4).

Table 4. Amounts of Kelthane produced in four resistant strains at two DDT doses.

Resistant strain	Kelthane content in μg/g			
	No. of samples	200μg DDT/ml	No. of samples	1000μg DDT/ml
Hikone-R	7	119±12	8	257± 35
WMB	6	278±28	7	379± 26
WMH	4	393±22	4	326± 59
TDE-R	3	519±75	5	598±113

D. Genetical Aspects of DDT-Metabolism

This section reports a study of the resistance problem from the joint approach of genetics and biochemistry. As shown above, DDT-resistant strains can metabolize absorbed DDT into Kelthane.

To ascertain the biochemical characteristic of the DDT-resistant gene *Rst(2)DDT*[5], F_1-hybrids between resistant and susceptible strains were first analysed for their ability to metabolize DDT. The Hikone-R and WMB strains were used as the resistant, and the Canton-S and *cn bw* strains were used as the susceptible ones in crossing experiments. Susceptible strains cannot survive at higher doses of DDT and hence it was impossible to examine their metabolism at such doses. Fortunately, since the resistant gene is dominant over the susceptible allele the F_1-hybrids are also resistant to DDT and hence they can metabolize it.

Oviposition was always obtained on DDT-free media, and the hatched first-instar larvae were collected and bred on DDT-containing media. Table 5 shows the results obtained from the reciprocal crosses between the resistant and the susceptible strains. When the susceptible strain was used as the female parent, the Kelthane content in the hybrid seemed to be slightly less than that in the hybrid from the resistant female. However, differences between each reciprocal cross or between hybrids and their parental resistant strain are not significant or scarcely significant at 5% level at 200μg/ml dose of DDT. At 1000μg/ml

Table 5. Comparison of Kelthane formation in the resistant and the F_1-hybrids at two doses of DDT.

Strain or cross	Kelthane content in μg/g			
	No. of samples	200μg DDT/ml	No. of samples	1000μg DDT/ml
Hikone-R	7	119±12	8	257±35
Hikone-R♀×Canton-S♂	3	114±27	3	134±33
Canton-S♀×Hikone-R♂	4	93±26	5	121±14
WMB	6	278±28	7	379±26
WMB♀×*cn bw*♂	7	295±30	—	—
cn bw♀×WMB♂	6	247±16	—	—

dose the difference in Kelthane content is highly significant between the hybrids and the parental Hikone-R strain.

Another genetical approach has been attempted to confirm the relation between resistance and metabolism by using special synthesized strains: HR-1, HR-2 and HR-3. These strains have a common susceptible chromosomal make-up or genetical background except for the particular chromosome by which these strains are characterized.

Synthesis of these special strains has been performed by chromosomal substitutions using a chromosome on DDT-resistance was proved to be negligible[5], its constitution was ignored.

The major DDT-resistant gene, *Rst(2) DDT*, is located on the second chromosome of the resistant strain, and hence only the HR-2 strain is resistant to high doses of DDT among these special strains. Resistance patterns of these strains to some insecticides are summarized in Table 6. In this table it is obvious that the second chromosomal factor from the Hikone-R strain is responsible for resistance to DDT, Dipterex and Diazinon, and it is also evident that resistance to nicotine sulfate is mainly due to the third chromosomal

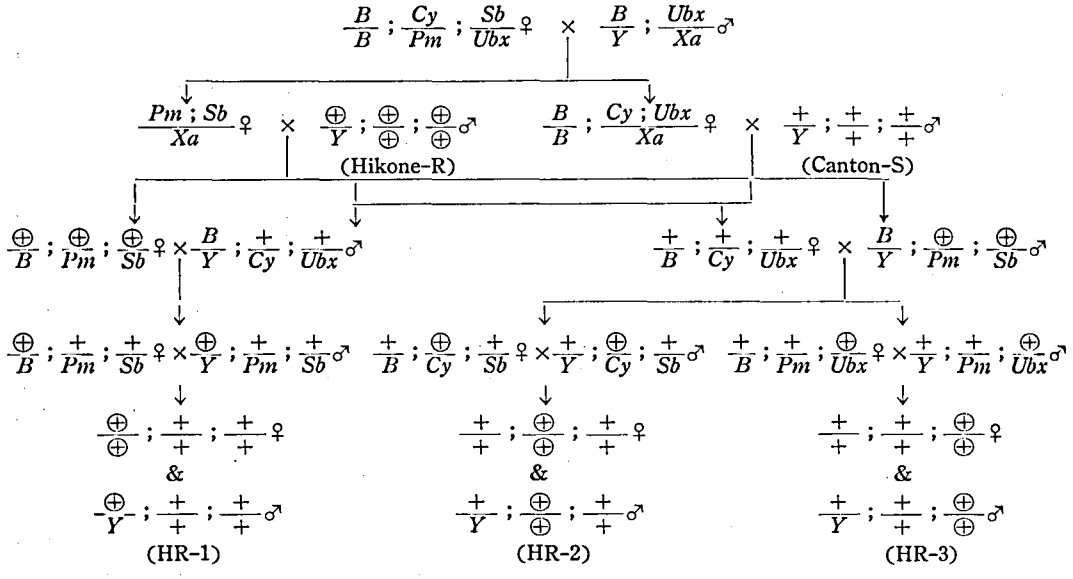


Fig. 1. Crossing procedures for synthesis and chromosomal constitutions of special strains
+ : chromosomes derived from the susceptible strain
⊕ : chromosomes derived from the resistant strain

multichromosomal mutant strain. The crossing procedures and chromosomal constitutions of special strains are illustrated in Figure 1. The exact description of this non-resistant multichromosomal mutant strain is as follows: *sc*^{v2} *B y*^{sP}; *Ins SM1, al Cy sp*^{2}*/dp b Pm* ; *In (3L) P, In(3R) C, Sb e l (3) e/Ubx*^{130} *e*^{s}. All the major chromosomes are marked with dominant mutations, except for the smallest fourth chromosome, and carry the large chromosomal inversion by which crossing-over between heterozygous chromosomes is suppressed. Since the effect of the fourth factor, this confirming previous results[4,7].

To compare the ability of the Kelthane production of susceptible and resistant strains at the same dose, the relatively low dose of DDT, 30 μg/ml, was employed. At this concentration, even the highly susceptible Catnon-S strain can produce apparently normal pupae, but the adult flies cannot emerge normally.

Quantitative analyses for Kelthane production were carried out by the Fujiwara reaction with only 5.0 ml. pyridine and 3.0 ml. NaOH solution in this instance, since the amounts of produced

Table 6. Resistance patterns of the synthesized strains to several insecticides. Data are expressed in number of adults emerged from larvae reared on insecticide-containing media, with the percent emergence in parentheses.

Insecticide	Dosage	No. of larvae tested for each strain	No. (and %) of adults emerged		
			HR-1	HR-2	HR-3
DDT	500 μg/ml	300	1 (0.3)	257 (85.7)	5 (1.7)
	1000	500	0 (0.0)	418 (83.6)	1 (0.2)
	2000	200	0 (0.0)	143 (71.5)	0 (0.0)
Dipterex	7.0 ppm	200	0 (0.0)	121 (60.5)	0 (0.0)
Diazinon	0.4 ppm	300	0 (0.0)	46 (15.3)	1 (0.3)
Nicotine sulfate	400 ppm	200	38 (19.0)	53 (26.5)	178 (89.0)
	600	300	4 (1.3)	31 (10.3)	125 (41.7)
	1000	300	0 (0.0)	0 (0.0)	30 (10.0)

Table 7. Kelthane production in the susceptible, mutant, resistant and synthesized strains at a DDT dose of 30 μg/ml.

Strain	Relative susceptibility to DDT	No. of samples	Kelthane content in pupae in μg/g
Canton-S	highly susceptible	3	3.3 ± 6.3
cn bw	susceptible	5	31.5 ± 4.2
bw; st; sv^{n}	less susceptible	4	25.7 ± 4.7
Hikone-R	resistant	6	33.5 ± 17.5
HR-1	susceptible	3	2.3 ± 1.0
HR-2	resistant	4	40.1 ± 11.0
HR-3	susceptible	3	5.3 ± 4.0

metabolite were expected to be extremely small in susceptible strains. Table 7 presents results with the resistant, non-resistant and some mutant strains at a DDT dose of 30 μg/ml.

These results apparently indicate that non-resistant strains are capable of metabolizing DDT to Kelthane in small amounts, and that there is no significant difference in Kelthane content between the susceptible *cn bw*, the DDT-tolerant *bw; st; sv*^{n} and the DDT-resistant Hikone-R and HR-2 strains at this sublethal dose of DDT.

Discussion

From the results presented in these tables, it may be concluded 1) that Kelthane is present in larvae, pupae and adults of *Drosophila* after they have developed on DDT-containing media, 2) that the amounts of Kelthane produced in pupae are not directly proportional to the resistance levels of the strains tested, especially at a low dose of DDT, 3) that F_1-hybrids between the resistant and susceptible strains are also capable of producing large amounts of Kelthane, and 4) that the greater Kelthane formation is correlated with the resistant second chromosomal factor.

The first purpose of this investigation, to present a quantitative basis for the DDT-Kelthane conversion, has been accomplished by these results. The second purpose, however, of ascertaining a relationship between the metabolism and the resistance or resistant gene has proved extremely difficult from these data obtained exclusively from *in vivo* experiments.

Several possible biochemical mechanisms for insecticide-resistance have been proposed by many workers on the basis of observed differences between resistant and susceptible strains. Most of the differences have been due merely by chance to the genetic origin of the strains used, and no hypothesis has proved consistent for all strains examined except one based on the metabolism of DDT by DDT-resistant houseflies. Indeed, dehydrochlorination, at that time the only identified metabolic pathway of DDT in insects, has been consistently found to be more rapid in resistant than in susceptible strains of houseflies.

Furthermore, *in vitro* experiments with housefly homogenates or enzyme preparations also supported such a correlation: the more resistant the strain, the greater the DDT-DDE conversion. There were some objections to this mechanism: an enhanced metabolism in resistant insects could be not a cause of resistance but a consequence of resistance, since resistant insects can survive longer and are physiologically more vigorous than non-resistant insects after treatment with the insecticide. Since the results of *in vitro* experiments have negated such an objection, the hypothesis that the detoxifying metabolism of an insecticide is one of the major mechanisms for insecticide-resistance is still valid at the present time. Genetic studies on DDT-resistance and DDT-dehydrochlorinase activity by Lovell & Kearns[2] or on organophosphate-resistance and ali-esterase activity by Oppenoorth[3] in the housefly are good examples of the most convincing experiments which reveal the relation between the *gene* for enzyme activity and the *gene* for resistance, independently of the *strains* used.

In the present investigation on *Drosophila* metabolism, only Kelthane was determined, using the Fujiwara reaction, since technical difficulties were associated with analyses for other compounds. Therefore, it is not to be expected that the dynamic pattern of the DDT-metabolism in *Drosophila* is adequately reflected by quantitative analyses of Kelthane in pupal samples alone. It is still uncertain whether Kelthane is a primary metabolite or metabolic pathway, since the water-soluble fraction was not analysed in this investigation. If the production of Kelthane in resistant strains is followed by its rapid degradation or excretion, determinations of Kelthane alone would seldom be proportional to the level of resistance. Unusually large differences in Kelthane content between DDT-resistant strains suggest the necessity of further experiment. One should, therefore, restrain from concluding whether the mesabolism is a cause or a consequence of the resistance and whether the metabolism is due to a biochemical function of the resistant gene, until more detailed balance-sheets on the metabolism are available.

For this purpose it is necessary to perform enzymatic experiments *in vitro* in which comparisons will be made independent of considerations of mortality, especially in susceptible strains. Preliminary studies *in vitro* are currently being re-attempted, although it was found that neither DDE nor Kelthane was produced from DDT by homogenates or enzyme preparations of *Drosophila* under conditions favourable for the housefly enzyme, DDT-dehydrochlorinase.

Acknowledgment

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Résumé

L'auteur donne le résultat des recherches quantitatives et génétiques sur le métabolisme du DDT chez les souches de drosophile sensibles et résistantes aux insecticides. La comparaison de la production du Kelthane *in vivo* a été faite selon la réaction de Fujiwara après le traitement avec le DDT. Les souches sensibles et résistantes et leurs hybrides peuvent convertir du DDT au Kelthane, mais les quantités du Kelthane qui se produit dans les drosophiles ne sont pas proportionnelles directement avec les degrés de larésistance des souches au DDT.

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