Toxicokinetic Analysis of Dermally Applied Diazinon in Resistant and Susceptible Houseflies, *Musca domestica* L.

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Toxicokinetic analysis was employed to investigate significance of various factors responsible for resistance in susceptible and resistant strains of housefly to diazinon. Topical application and application by exposure to a residual film of the compound in holding vial was conducted. A significant difference was observed between the strains in the penetration rate of diazinon, the amount of diazoxon present, and the degree of AChE inhibition. The data also indicated a reversible exchange of diazinon between external surface of the flies and the holding vial by volatilization/rub-off and resorption of the compound by the flies. Certain k values estimated by computer simulation using the experimental data and differential equations derived from the toxicokinetic models proposed for the external and internal dynamics of diazinon demonstrated an inter-strain difference. In particular, the ratio of diazoxon degradation over activation of diazinon was much greater in the resistant strain than in the susceptible strain, suggesting that low accumulation of diazoxon was the most important resistance factor. Insensitive AChE also played an important role *in vivo*.

Key words: Toxicokinetics, diazinon, housefly, resistance, mathematical model

INTRODUCTION

Studies on the mechanism responsible for insecticide resistance generally involve the *in vitro* comparison of various biochemical factors present in resistant and susceptible strains of insects. However, an *in vitro* study does not provide information on the actual significance of the factors *in vivo*. To investigate the significance, toxicokinetic study is useful.

The toxicity of a compound is the result of complex interactions of a variety of toxicological processes such as penetration, activation, degradation, distribution, binding to internal tissues, action at the target site, etc. Quantitative evaluation of all these processes and their interactions can be described by toxicokinetic parameters.

An attempt to introduce toxicodynamic models for insecticidal action began in the late 1960s. SUN (1968) evaluated the relationship between the relative toxicity of insecticides and the rates of penetration and detoxication. Subsequently, several

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papers appeared concerning the toxicokinetic studies of insecticides (for review, see WELLING and PATERSON, 1985). Recently, physiological models were used to account for differences in concentration of insecticide between individual tissues in larvae of *Spodoptera littoralis* (GREENWOOD et al., 1990).

With regard to the analysis of resistance mechanisms, few studies employed toxicokinetic approaches (see HOLLINGWORTH, 1971 for fenitrothion and WELLING et al., 1983 for malaoxon resistance of housefly.). These studies include simplified models consisting of linear rate equations.

This paper is a part of a series of toxicokinetic studies for the evaluation of resistance factors *in vivo*. The studies deal with a Yachiyo strain of diazinon-resistant housefly. Biochemical factors responsible for the resistance are increased activities in cytochrome P-450 dependent monooxygenases (MFO), glutathione transferase (GST), and phosphotriester hydrolases, as well as insensitivity at the target enzyme, AChE (OI et al., 1990). The purpose of this paper is to compare the *in vivo* dynamics and kinetic parameters of diazinon from topical application until AChE inhibition in a resistant and a susceptible strain of housefly.

MATERIALS AND METHODS

Houseflies. CSMA and Yachiyo strains used are the same strains as described previously (OI et al., 1990). LD₅₀ value of diazinon determined by topical application were 0.035 µg and 50 µg per female fly, respectively.

Chemicals. [Ethoxy-¹⁴C]diazinon, *O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate (sp. act. 1.8 µCi/mg) (>99% radiochemical purity), and nonradioactive diazinon and diazoxon, *O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphate, were the same as described previously (OI et al., 1990). The nonradioactive diazinon and diazoxon were purified before use. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ATCh) were purchased from Kanto Chemical Co., Inc.

Dynamics of ¹⁴C-diazinon. In order to study the dynamics of ¹⁴C-diazinon, the insecticide was applied to three- to six-day-old female flies by two methods. The first was by topical application with 0.5 µl of acetone solution of diazinon applied on the thoracic scutum. Treated flies were kept in a 20 ml scintillation vial at 25°C. The second method was residual application in which flies were held in a scintillation vial pre-coated with a residual film of diazinon prepared by evaporation of the acetone solution of diazinon. Flies were kept either continuously exposed to the residual film or were transferred to a clean vial after 30 min exposure to the residual film. A group of ten flies were treated by both methods with three replications.

At various time intervals after treatment, flies were rinsed with glass filter by pouring 5 ml portions of chloroform four times to remove the external diazinon (external rinse). The flies were then homogenized in a mixture of chloroform (4 ml) and water (2 ml). The homogenate was stirred vigorously and then centrifuged to separate the chloroform (chloroform-soluble internal extract) and water (water-soluble internal extract) layers. The vial which held the flies was washed with chloroform (2 ml) and water (2 ml) and partitioned as described above to separate water-soluble excreta and diazinon volatilized and removed from the flies. Chloroform solutions were transferred to a scintillation vial and the solvent was evaporated under a stream

of nitrogen following the addition of a drop of Triton X-100 and ethanol containing 20% KOH to prevent the loss of diazinon by evaporation. The radioactivity in the various fractions was determined by a liquid scintillation spectrophotometer (LSC 65, Aloka Co. Ltd., Tokyo, Japan) after the addition of scintillation cocktail (PATERSON and GREEN, 1965). In preliminary study, radioactivity detected in the housefly residue was less than 2% (ca. 80 DPM) of total radioactivity in both strains when 0.1 μg /female was applied topically. With the residual film method, flies were homogenized in the scintillation cocktail after removal of the external diazinon (internal radioactivity). The radioactivity recovery was greater than 78% throughout the experiments, and the mean value of the recovery was more than 90% of applied dose.

Biochemical determination of diazoxon. The amount of diazoxon formed following diazinon application was determined by measuring the amount of inhibition of housefly head AChE from the CSMA strain. A crude homogenate (five heads per ml of 0.1 M phosphate buffer, pH 8.0) filtered through glass wool was used as the AChE solution. A portion of chloroform extract obtained from the flies after removal of external diazinon as described above was pre-incubated after evaporation of the solvent with the AChE solution at 37°C for either 5 min or 20 min, depending upon the concentration of diazoxon. The reaction mixture consisted of 0.05 ml of AChE solution, 2.85 ml of 0.1 M phosphate buffer, pH 8.0, 0.05 ml of 0.02 M DTNB, and diazoxon. After the pre-incubation, 0.05 ml of 0.1 M ATCh was added and the remaining AChE activity was determined at 37°C according to ELLMAN et al. (1961). Percent inhibition of AChE activity was converted to ng diazoxon using calibration curves described as follows,

$$\begin{aligned} Y &= 5 + 1.9718 (X_1 + 0.0124) && \text{(pre-incubation: 5 min)} \\ Y &= 5 + 1.7537 (X_1 + 0.6604) && \text{(pre-incubation: 20 min)} \end{aligned}$$

where Y is probit of percent inhibition of AChE and X_1 is the common logarithm of ng diazoxon in the reaction mixture. Since the chloroform extract of the flies contained not only diazoxon but also a considerable amount of internal diazinon, the effect of diazinon on AChE inhibition was corrected using a calibration curve similarly prepared for diazinon,

$$Y = 5 + 1.7833 (X_2 - 1.0954) \quad \text{(pre-incubation: 5 min)}$$

where X_2 is the common logarithm of ng diazinon in the reaction mixture. The amount of internal diazinon was estimated from a separate experiment using radio-labeled diazinon.

Time-course of AChE inhibition. Residual AChE activity in the head of flies treated topically with diazinon was determined immediately after the preparation of AChE with progression of time using the method mentioned above. The possible secondary inhibition of AChE by free diazoxon in the head was avoided by including 2.5 mM ATCh in the homogenization buffer to protect the free AChE.

Estimation of kinetic parameters by computer simulation. Kinetic parameters for models proposed in the text were estimated by computer simulation using the differential equations and experimental data. Computer simulation was carried out on a personal computer (NEC 9801) with commercially available programs (Nankoudou, Inc.) which was based on the previous paper (YAMAOKA et al., 1981). In estimation of parameter k , least squares method was employed according to the following equation:

$$SS = \sum W_i (C_i - f(t_i, P))^2$$

where SS , C_i , and $f(t_i, P)$ are sums of squares, measured value at time t_i , and simulated value at time t_i with parameter value P . 1, $1/C_i$ or $1/C_i^2$ can be used as the weight value, W_i . In this study, the weight value of 1 was used because the experimental error of larger values were less than that of smaller values.

RESULTS

Dynamics of topically applied diazinon

The following experimental results are the average of three measurements. The ratio of radioactivity in each compartment was expressed based upon the total radioactivity recovered. Distribution of radioactivity following the topical application of ^{14}C -diazinon at various doses is shown in Fig. 1. Doses applied were 0.02 and 0.1 $\mu\text{g}/\text{female}$ (approximately equivalent to LD_{30} and LD_{90} , respectively) for the CSMA strain, and 0.02, 0.1, 0.5 and 5 $\mu\text{g}/\text{female}$ (below lethal doses) for the Yachiyo strain. The radioactivity in the water-soluble internal extract was combined with the excreta wash extracted from the holding vial and shown together in the figures.

Disappearance of diazinon from the external surface of the flies was faster in the

Fig. 1. Distribution of diazinon applied topically to CSMA (S) and Yachiyo (R) strains. Strains and applied doses are shown in the figures. ●: external rinse; ○: chloroform-soluble vial wash; △: chloroform-soluble internal extract; ▲: sum of water-soluble internal extract and excreta. The curves were obtained by computer simulation based on the rate constants listed in Table 1.

CSMA strain than in the Yachiyo strain at 0.02 and 0.1 $\mu\text{g}/\text{female}$. In the Yachiyo strain, to which higher doses were also applied, the ratio of disappearance decreased at higher doses.

The chloroform-soluble radioactivity recovered from the vial wash appeared to be slightly larger in the Yachiyo strain than in the CSMA strain. The radioactivity is diazinon transferred to the holding vial from the external surface of the flies via volatilization and rub-off, because it was not found when ^{14}C -diazinon was injected into the flies and only water-soluble radioactivity was recovered from the holding vial in a separate experiment.

The chloroform-soluble radioactivity recovered from the internal extract was higher in the CSMA strain than in the Yachiyo strain at 0.02 and 0.1 $\mu\text{g}/\text{female}$. The inter-strain difference was larger at the higher dose.

The amount of water-soluble radioactivity recovered from the internal extract and the vial wash was larger in the CSMA strain than in the Yachiyo strain within the first 2 h after application. Although the results seem to suggest a higher detoxication ability in CSMA strain, it could be due to faster penetration of diazinon.

The amount of diazinon recovered from the holding vial decreased after reaching its maximum, suggesting a possible resorption of the compound through contact with the external surface of the flies. Therefore, an attempt was made to investigate the dynamics of diazinon transferred to the holding vial from the external surface of the flies by volatilization or rub-off.

Dynamics of diazinon applied by exposure to the residual film

The flies were allowed to have free contact with the residual film of ^{14}C -diazinon and the distribution of the radioactivity was studied over time (Fig. 2). The sum of radioactivity recovered from the internal extract and the water-soluble fraction of excreta was regarded as the total amount penetrated. The rate of disappearance of

Fig. 2. Distribution of diazinon by continuous contact with residual film of diazinon in CSMA (S) and Yachiyo (R) strains. Strains and applied doses are shown in the figures. ●: external rinse; ○: chloroform-soluble vial wash; △: total amount penetrated (sum of internal extract and water-soluble excreta). The curves were obtained by computer simulation, based on the rate constants listed in Table 1.

Fig. 3. Distribution of diazinon resorbed by prior contact with residual film of diazinon for 30 min in CSMA (S) and Yachiyo (R) strains. Strains and amount of diazinon present on the external surface at zero time, when houseflies were transferred to the clean vial after prior contact with residual film, are shown in the figure. ●: external rinse; ○: chloroform-soluble vial wash; △: total amount penetrated (sum of internal extract and water-soluble excreta). The curves were obtained by computer simulation, based on the rate constants listed in Table 1.

diazinon from the holding vial was faster in the CSMA strain than in the Yachiyo strain. A parallel pattern was the higher rate of penetration in the CSMA strain than in the Yachiyo strain when compared at the same dose of $0.2 \mu\text{g}/\text{vial}/10$ females. Exposure of the Yachiyo strain to the higher doses, i.e. 5 and $50 \mu\text{g}/\text{vial}/10$ females, did not show dose-dependency for diazinon.

In order to investigate the dynamics of diazinon picked up on the external surface of the flies, the flies were exposed to a residual film of ^{14}C -diazinon for 30 min and then transferred to a clean vial (Fig. 3). Data was based on the total radioactivity recovered. After prior contact with the residual film of ^{14}C -diazinon at $1 \mu\text{g}/\text{vial}/10$ females for the CSMA strain and 1, 5 and $50 \mu\text{g}/\text{vial}/10$ females for the Yachiyo strain, the amounts of diazinon present on the external surface of the flies were 0.011, 0.014, 0.065 and $0.5 \mu\text{g}/\text{female}$, respectively. Data in Fig. 3 show that the rate of disappearance of diazinon from the external surface of the flies was slower and the amount of chloroform-soluble radioactivity transferred to the holding vial was larger with the Yachiyo strain than with the CSMA strain. Consequently, diazinon penetration was lower in the Yachiyo strain than in the CSMA strain. These results are similar to those obtained when diazinon was applied to the flies topically (Fig. 1). Results obtained at higher doses with the Yachiyo strain again suggest that these processes are not dose-dependant. Figures 2 and 3 confirm reversible exchange of diazinon between the external surfaces of the flies and the holding vial.

Time-course of diazoxon following topical application of diazinon

The biochemically-determined fate of diazoxon following topical application of diazinon is shown in Fig. 4. At doses applied to CSMA strain, i.e. 0.02 and $0.1 \mu\text{g}/$

Fig. 4. Time-course of internal diazoxon following topical application of diazinon in CSMA (S) and Yachiyo (R) strains. Diazinon applied to CSMA strain, 0.02 $\mu\text{g}/\text{female}$ (●) and 0.1 $\mu\text{g}/\text{female}$ (○), resistant strain, 5 $\mu\text{g}/\text{female}$ (●) and 50 $\mu\text{g}/\text{female}$ (○). The curves were obtained by computer simulation, based on rate constants listed in Table 1, except the dotted curve in resistant strain which was obtained by the method of residuals.

female, no diazoxon was detected in the Yachiyo strain. Therefore, much higher doses, i.e. 5 and 50 $\mu\text{g}/\text{female}$ were applied to the Yachiyo strain. The internal diazinon was not determined at 50 $\mu\text{g}/\text{female}$. Since the data obtained at this dose were not corrected for possible interference by internal diazinon, they indicate only an apparent amount of diazoxon.

The percent of diazoxon in the CSMA strain reached its maximum 30 min after treatment, diazoxon being approximately 3% of the diazinon applied. Thereafter, diazoxon gradually decreased with time at both diazinon dosage levels. The percent of diazoxon found in the CSMA strain was higher when the flies were treated with the higher doses of diazinon.

In the Yachiyo strain, the percent of diazoxon formed was much smaller than that in the CSMA strain, the maximum being below 0.008% at 50 $\mu\text{g}/\text{female}$ and 0.03% at 5 $\mu\text{g}/\text{female}$. No significant difference was observed between the two doses in terms of the absolute amount of diazoxon present up to 2 h after the treatment, despite a ten-fold difference in the amount of diazinon applied.

Time-course of AChE inhibition following topical application of diazinon

Two doses of diazinon, 0.02 μg and 0.1 $\mu\text{g}/\text{female}$, were applied to the CSMA strain. The higher dose resulted in more inhibition of the target enzyme. As shown in Fig. 5, although the flies received diazinon topically on the thorax, AChE inhibition in the head appeared to correlate well with the progress of the toxic symptoms. Over 90% of the flies were paralyzed when 50% inhibition of AChE occurred. On the other hand, with the Yachiyo strain, a large dose (50 $\mu\text{g}/\text{female}$) was needed, since at 5 μg or less no significant inhibition of AChE was observed up to 4 h after treatment.

Kinetic analysis of external dynamics of diazinon and degradation.

Based upon the experimental data shown in Fig. 1–3, the external dynamics of topically applied diazinon are described in Fig. 6. The following differential equations are derived from the model by linear approximation:

Fig. 5. Time-course of residual AChE activity in the head following topical application of diazinon in CSMA (S) and Yachiyo (R) strains. Diazinon applied to CSMA strain, 0.02 $\mu\text{g}/\text{♀}$ (●) and 0.1 $\mu\text{g}/\text{♀}$ (○); Yachiyo strain, 50 $\mu\text{g}/\text{♀}$. The curves were obtained by computer simulation, based on rate constants listed in Table 1.

Fig. 6. Kinetic model describing the fate of topically applied diazinon. X_1 : diazinon on the thorax; X_2 : diazinon attached to the vial by volatilization and rub-off; X_3 : diazinon resorbed by external surface of the flies; X_c : Chloroform-soluble internal diazinon and diazoxon; X_w : water-soluble metabolites and excreta.

$$\frac{dX_1}{dt} = -(k_{12} + k_{14}) X_1 \quad (1)$$

$$\frac{dX_2}{dt} = k_{12} X_1 - k_{23} X_2 + k_{32} X_3 \quad (2)$$

$$\frac{dX_3}{dt} = k_{23} X_2 - (k_{32} + k_{34}) X_3 \quad (3)$$

$$\frac{dX_c}{dt} = k_{14} X_1 + k_{34} X_3 - k_{cw} X_c \quad (4)$$

$$\frac{dX_w}{dt} = k_{cw} X_c \quad (5)$$

where X_1 , X_2 , X_3 , X_c and X_w are described in Fig. 6. Rate constants for the processes are indicated by arrows and are expressed by k .

To estimate the parameter values accurately, the model should be simple. Therefore, parameter values k_{23} , k_{32} , and k_{34} were first estimated by computer simulation using the experimental data shown in Figs. 2 and 3. Since initial condition of these figures are X_2 and X_3 , respectively, the kinetic pathways of k_{12} and k_{14} can be neglected. Therefore equations 1–5 are simplified to equations 3, 6 and 7.

$$\frac{dX_2}{dt} = -k_{23} X_2 + k_{32} X_3 \quad (6)$$

$$\frac{dX_B}{dt} = k_{34} X_3 \quad (7)$$

where X_B is the total amount of penetrating diazinon in the experiments shown in

Figs. 2 and 3. The two initial conditions of X_2 and X_3 were combined and used for the estimation. Since there was no dose-dependency of the rate constants under the experimental condition in the Yachiyo strain, the mean values obtained from the three doses were used for the following simulation.

The k_{12} , k_{14} , and k_{CW} values were then estimated by computer simulation using the experimental data shown in Fig. 1, the k_{23} , k_{32} , and k_{34} values, and equations (1)–(5). All the parameter values thus estimated are listed in Table 1. Based upon these parameter values, the dynamics of diazinon were predicted and presented along with the experimental data in Figs. 1–3, which showed a fairly good agreement.

A comparison of k values between the two strains shows that diazinon penetration (k_{14} , k_{34}) and diazinon resorption by the external surface of the flies (k_{23}) in the CSMA strain was about twice as large as those from the Yachiyo strain. With regard to the transfer of diazinon from the external surface of the flies to the holding vial by volatilization and rub-off (k_{12} value) at the high dose $0.1 \mu\text{g}/\text{female}$, the inter-strain difference is probably due to the fact that the dose applied was approximately equivalent to the LD_{90} for the CSMA strain and caused knockdown of most of the flies. This could increase the amount of diazinon transferred to the vial by rub-off. The k value for detoxication (k_{CW}) was higher in the CSMA strain than in the Yachiyo strain at $0.02 \mu\text{g}/\text{female}$, but the relationship was reversed at $0.1 \mu\text{g}$. A decrease in the k_{12} , k_{14} , and k_{CW} values in the Yachiyo strain in response to the increase of the applied dose suggested dose dependency in these processes.

Kinetic analysis of activation, degradation, and AChE inhibition

The fate of diazinon after penetration can be described by the model depicted in Fig. 7. The following differential equations were derived from the model by linear approximation:

$$dX_4/dt = k_{14} X_1 + k_{34} X_3 - (k_{45} + k_{46}) X_4 \quad (8)$$

$$dX_5/dt = k_{45} X_4 \quad (9)$$

$$dX_6/dt = k_{46} X_4 - k_{67} X_6 \quad (10)$$

$$dX_7/dt = k_{67} X_6 \quad (11)$$

where X_4 , X_5 , X_6 , X_7 and rate constants expressed by k are described in Fig. 7. Since the rate of AChE inhibition depends upon both the internal amount of diazoxon and the residual activity of AChE, it is a bimolecular reaction which can be expressed by the equation:

$$dE_A/dt = -k_{6A} X_6 E_A \quad (12)$$

where E_A is AChE. This equation is similar to that described by ALDRIDGE (1950), although the parameter k_{6A} is different from the bimolecular rate constant, k_i , in that X_6 is an absolute amount, not a concentration.

The kinetic parameters governing the internal dynamics of diazinon should not be estimated at the same time, because the model is too complex and data on time-course of internal diazinon and diazoxon and AChE inhibition were obtained in separate experiments. Therefore, the following procedure was employed to estimate those values. First, the value for the disappearance of diazinon k_s , which is defined as the sum of k_{45} and k_{46} in the model depicted in Fig. 7, was estimated by computer simulation using the data on the internal amount of diazinon (estimated by subtracting the

Fig. 7. Kinetic model describing the internal fate of diazinon following penetration. X_4 : internal diazinon; X_5 : water-soluble metabolites of diazinon; X_6 : internal diazoxon; X_7 : water-soluble metabolites of diazoxon; E_A : AChE; PX : diazoxon (P and X denote diethyl phosphoryl and pyrimidine groups, respectively).

Table 1. Kinetic parameters for the model given in Figs. 6 and 7

Parameter ^a	Dose ($\mu\text{g}/\text{♀}$)	Parameter value (h^{-1}) $\pm$ S.D.	
		S	R
k_{23} (resorption)		1.11 ± 0.13	0.606 ± 0.039
k_{32} (volatilization and rub-off)		1.04 ± 0.20	0.917 ± 0.070
k_{34} (penetration)		1.52 ± 0.13	0.706 ± 0.034
k_{12} (volatilization and rub-off)	0.02	0.689 ± 0.117	0.747 ± 0.072
	0.1	0.866 ± 0.100	0.462 ± 0.045
	0.5	N.D. ^b	0.501 ± 0.034
	5	N.D.	0.114 ± 0.007
k_{14} (penetration)	0.02	1.88 ± 0.14	0.998 ± 0.071
	0.1	2.05 ± 0.12	0.881 ± 0.049
	0.5	N.D.	0.384 ± 0.025
	5	N.D.	0.104 ± 0.006
k_{0W} (detoxication)	0.02	3.74 ± 0.51	2.66 ± 0.41
	0.1	1.92 ± 0.14	3.55 ± 0.53
	0.5	N.D.	3.40 ± 0.76
	5	N.D.	1.41 ± 0.19
k_8 (disappearance of diazinon) ^c	0.02	3.71 ± 0.40	N.D.
	0.1	2.17 ± 0.20	N.D.
	5	N.D.	1.48 ± 0.22
k_{46} (activation)	0.02	0.681 ± 0.268 (1)	N.D.
	0.1	0.538 ± 0.092 (1)	N.D.
	5	N.D.	0.0158 ± 0.0074 (1)
k_{67} (detoxication of diazoxon)	0.02	5.56 ± 2.61 (8.2)	N.D.
	0.1	3.79 ± 0.79 (7.0)	N.D.
	5	N.D.	4.36 ± 2.17 (276)
k_{6A} (inhibition of AChE) ^d	0.02	584 ± 178	N.D.
	0.1	178 ± 24	N.D.
	50	N.D.	53.5 ± 7.8

^a The procedure of parameter estimation is described in the text.

^b Not determined

^c $k_8 = k_{45} + k_{46}$

^d The unit is $\text{h}^{-1} \cdot \mu\text{g}^{-1}$

internal amount of diazoxon from the internal amount of chloroform-soluble radioactive compounds), equations (1)–(3) and (8), and values already determined for the external dynamics of diazinon (Table 1).

Second, the rate constant values for activation k_{46} and degradation of diazoxon k_{67} were estimated by computer simulation using the data from Fig. 4 of diazoxon, equations (1)–(3), (8) and (10), and parameter values already determined (k_{12} , k_{14} , k_{23} , k_{32} , k_{34} and k_s). Finally, the inhibition constant for AChE, k_{6A} , was estimated by computer simulation using the time-course data of AChE inhibition (Fig. 5), equations (1)–(3), (8), (10) and (12), and parameter values already determined (k_{12} , k_{14} , k_{23} , k_{32} , k_{34} , k_s , k_{46} and k_{67}).

The estimated k values are presented in Table 1 along with the other parameter values already determined. Since k_{46} and k_{67} values were estimated with only a time-course of diazoxon but not with that of diazoxon metabolites, the values are easily subjected to the experimental error of the diazoxon time-course. The values are also accompanied by larger standard deviation than other parameter values. Therefore, a direct comparison of the k values between the strains is not possible. In order to evaluate the relative role of degradation of diazoxon and activation of diazinon to diazoxon, the ratio of k_{67}/k_{46} values were compared between the strains and expressed in parentheses in Table 1. The influence of the experimental error to the k_{67}/k_{46} values were much smaller than to k_{46} and k_{67} values. The results clearly show that the ratio in the Yachiyo strain is much higher than that in the CSMA strain, suggesting the possibility of little accumulation of diazoxon occurring in the resistant strain.

Since the time-course of ^{14}C -diazinon in the Yachiyo strain at the dose of 50 μg /female was not investigated, k_{6A} of Yachiyo strain was determined with the time-course of AChE inhibition (Fig. 5) and parameter values of fate of diazoxon simulated by the method of residuals (the simulated time-course of diazoxon is shown in Fig. 4). The method is expressed with sum of exponential functions to fit simulated data to experimental data. The fact that the value obtained in the Yachiyo strain at one dose was lower than those values obtained in CSMA strain at two different doses coupled with the fact that the bimolecular rate constant k_i determined *in vitro* was 5.9 times smaller in Yachiyo strain than in CSMA strain (Ori et al., 1990) implies an important role that insensitive AChE plays in diazinon resistance.

DISCUSSION

Among the metabolic reactions examined for diazinon resistance in the previous study (Ori et al., 1990), the MFO and GST from the Yachiyo strain degraded more diazinon *in vitro* than those from the CSMA strain. But, neither of these factors appear to play a vital role in resistance *in vivo*, judging from the results of this study. Only a small inter-strain difference was observed in the k_{CW} values (Table 1). In any case, the relative contribution of diazinon degradation *in vivo* remains to be studied, because k_{CW} is a parameter for overall degradation including diazinon and diazoxon, and the activation rate constant k_{46} was not obtained correctly in this study. On the other hand, the remarkable inter-strain difference observed in the k_{67}/k_{46} ratio can lead to little accumulation of the actual toxicant for AChE inhibition, diazoxon, and therefore would be the most important factor—particularly when coupled with insensitive AChE for diazinon resistance. Though k_{67} value was not obtained correctly in this study,

diazoxon degradation is probably an important factor of this resistance. This is indicated by the result that DEF, a phosphorotriester hydrolase inhibitor, showed remarkable synergism when combined with diazinon (OI and MOTOYAMA, 1991a).

This study also demonstrated a slower penetration of topically applied diazinon and that of diazinon picked up from residual film as other resistance factors in the Yachiyo strain (Figs. 1 and 3, and Table 1). The inter-strain difference in the rate was about two-fold as judged by the k_{14} and k_{34} values. A reduction in penetration as a resistance mechanism in the housefly has long been known (FORGASH et al., 1962) and is associated with a difference in the total quantity and composition of the cuticular lipids (PATIL and GUTHRIE, 1978). The inter-strain difference was also observed in parameter values for resorption, k_{23} , when flies were exposed to the residual film of diazinon. Since diazinon applied by this method is assumed to make contact with the flies on the external surface of the extremities, our results suggest alteration in the cuticular lipids causing a slower penetration of diazinon not only on the thoracic scutum but also on the extremities.

Dose dependency of penetration was indicated in epidermal tissue of common cutworm, and was evaluated by kinetic approaches (OI and MOTOYAMA, 1991b). Dose dependency was also indicated in this study as was shown by a decrease in the k_{14} value in accordance with an increase in the dose of diazinon applied. This dose-dependency indicates a limited capacity for the penetration process, which may result in an artificially high resistance ratio due to the saturation which occurs when extremely large doses are applied to determine the LD_{50} .

With the Yachiyo strain, a significant inhibition of AChE was observed 4 h after the topical application with 50 $\mu\text{g}/\text{♀}$ but not with diazinon doses of 5 μg or less (Fig. 5). Since the internal amount of diazoxon found under the test conditions were around 3 and 1 $\text{ng}/\text{♀}$, respectively (Fig. 4), a lethal level of diazoxon for the Yachiyo strain appears to lie within this range. In support of this assumption, an increase in intoxication was observed between 2 to 4 h after topical application with 50 $\mu\text{g}/\text{♀}$ of diazinon. It is also noteworthy that a significant amount of AChE inhibition was observed in the CSMA strain when the amount of internal diazoxon was below 0.6 ng after the topical application with 0.02 μg of diazinon (Figs. 4 and 5). These results verify the important role that insensitive AChE plays in diazinon resistance in the Yachiyo strain.

Judging from the kinetic parameters listed in Table 1, the resistance factors (such as insensitive AChE) were in good agreement with the results obtained from *in vitro* study (OI et al., 1990), although diazinon degradation contradicted with the results. This suggested that *in vitro* studies are highly artificial. Toxicokinetic analysis therefore plays an important role in investigating actual significance of resistance factors *in vivo*. This study demonstrated the complicated dynamics of external diazinon. In this case, a simplified model obtained by changing application methods was available to estimate kinetic parameters.

Toxicokinetic evaluation of the significance of diazinon activation to diazoxon and degradation of diazoxon is still required. A detailed study of these processes are the subject of a subsequent study (in preparation).

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