Biochemical Pharmacology, 1965, Vol. 14, pp. 1011–1017. Pergamon Press Ltd., Printed in Great Britain.

INACTIVATION AND REACTIVATION RATES OF FLY AND BEE CHOLINESTERASES INHIBITED BY SEVIN*

RALPH E. KUNKEE† and GUNTER ZWEIG

Agricultural Toxicology and Residue Research Laboratory,
University of California, Davis, Calif., U.S.A.

(*Received* 20 *November* 1964; *accepted* 25 *January* 1965)

Abstract—The inactivation rate constants and the reactivation rate constants of insect cholinesterases inhibited by the carbamate insecticide Sevin (l-naphthyl N-methyl carbamate) were measured. The inactivation rate constant of honeybee cholinesterase, inhibited by Sevin, was five times larger than that of housefly cholinesterase. This difference in rate constants may explain the difference between bee and fly sensitivities to Sevin. The reactivation rate constants of the inhibited enzymes were about the same for both insects.

HOUSEFLIES and honeybees have different sensitivities to the carbamate insecticides such as Sevin.* The 24-hr LD_{50} for topical application of Sevin is only about 0·2 μg per bee but for flies it is much higher. LD_{50}'s ranging from 1·5 to 100 μg per fly have been reported[1–4] from various laboratories.‡ The toxicity has been attributed to the anticholinesterase activity of the carbamates.[5, 6] Since cholinesterase activity in the purified preparations from bees has different properties compared to that from flies,[7, 8] the species difference in sensitivity to Sevin may be a reflection of a difference in the enzymes. For these reasons we examined the inhibition of fly and bee cholinesterase by Sevin.

Sevin inhibits cholinesterase apparently by a carbamylation of the active site of the enzyme, analogous to phosphorylation by the toxic organophosphates.[6] However, carbamylated cholinesterase is unstable, and it is slowly reactivated by water; therefore special conditions are required to measure the extent of inhibition by carbamates. Wilson and co-workers have defined the conditions for the determination of both the

* l-Naphthyl N-methyl carbamate ("Sevin"; trademark registered by Union Carbide Chemicals Co.).

† Present address: Dept. of Viticulture and Enology, University of California, Davis, Calif.

‡ Houseflies and honeybees contain approximately equal amounts of cholinesterase; thus the LD_{50} can be compared on basis of insect. Specific differences between LD_{50}'s based on unit body weight would be even greater.

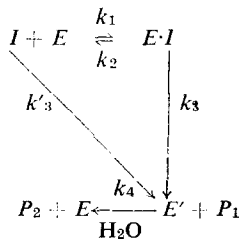
carbamylation (inactivation) of cholinesterase rate constant (k'_3) and the carbamyl–cholinesterase hydrolysis (reactivation) rate constant (k_4)[9–11]. Using these conditions,§ we have determined k'_3 and k_4 for cholinesterases of honeybees and houseflies. We find large differences in the inactivation constants of the enzymes, which may explain the differences in sensitivity of the insects to Sevin. For the results presented here, we used breis rather than purified preparations to obtain rate constants representing all the cholinesterases present. Only insects heads were used since most of the cholinesterase is found there.[12] (We are using "cholinesterase" in the general sense to include acetylcholinesterase and pseudocholinesterase).

MATERIALS AND METHODS

Houseflies (*Musca domestica L.*) were reared on a canned milk diet.[13] Honeybees (*Apis mellifera L.*) were obtained from the University Apiary and were collected under mild smoke anethesia. The insects were killed by freezing. Breis were prepared by grinding the adult insect heads with H_2O.[8]

Cholinesterase activity was determined colorimetrically with 5,5′-dithio*bis*-2-nitrobenzoic acid and acetylthiocholine.[8, 14] The assay mixtures were incubated for 10 min for the inactivation experiments and 5 min for the reactivation experiments.

§ Wilson and co-workers[9–11] treat the carbamyl derivative inhibitors as poor substrates:



where I is the inhibitor (Sevin), E is active cholinesterase, $E{\cdot}I$ is cholinesterase–inhibitor complex, E' is the carbamylcholinesterase (inactive), P_1 is one product (1-naphthol), and P_2 is the other product. Under conditions where $E{\cdot}I$ is in equilibrium with I and E, the inhibition rate can be characterized by k'_3. The reactivation rate is a pseudo first-order hydrolysis reaction which is characterized by k_4. The formation of inactive enzyme with time is:

$$\frac{dE'}{dt} = k'_3\,(I)(E) - k_4\,(E') \tag{1}$$

If (E) is small compared to (I), then (I) is essentially constant and a steady state is reached where:

$$\left(\frac{dE'}{dt}\right)_{ss} = 0 \text{ and } \left(\frac{E'}{E}\right)_{ss} = \frac{k_3}{k_4}\,(I)$$

Thus, under steady-state conditions, at 50% inhibition, $(I) = (I_{50})_{ss}$

$$k'_3 = \frac{k_4}{(I_{50})_{ss}}$$

The reactivation rate constant k_4 can be determined by a great dilution of a solution of the inhibited enzyme. This effectively makes $(I) = 0$ in equation (1) and gives:

$$\ln \frac{(E')}{(E'_0)} = -k_4 t$$

where (E'_0) and (E') are the concentrations of inhibited enzyme at zero time and at time t, respectively.

Eserine, final concentration 10^{-5} M, was added for determination of noncholinesterase activity corrections.[8, 15]

Inactivation rate constants were determined under steady-state conditions.§ Dilute breis (20 heads/ml diluted 500-fold for bee and 285-fold for fly with 0·1 M Na phosphate buffer, pH 8·0) were incubated with the indicated concentrations of Sevin at 30°. After various time periods, 3·6-ml samples were removed and assayed for cholinesterase activity. Activities of the uninhibited controls were also determined.

Reactivation rate constants were determined on bee- and fly-head breis treated with Sevin to give nearly complete inhibition and then greatly diluted. Two-tenths ml of brei (100 heads/ml) was incubated at 30° with 0·05 ml Sevin (1 μg/ml) for 30 min. The samples were then diluted 400-fold with 0·1 M Na phosphate buffer, pH 8·0, at 30° (zero time). After various time periods, 3·6 ml of the diluted samples was removed and assayed for cholinesterase. The concentration of inhibited enzyme (E') and the concentration of inhibited enzyme at zero time (time of dilution) (E'_0) were determined indirectly from the differences between the total cholinesterase activity (in absence of inhibitor) and the activity in the presence of the inhibitor. The ratio (E'/E'_0) was then calculated. (The *ratio* of enzyme concentrations is required;§ thus the concentrations can be given in arbitrary units.)

RESULTS

Inactivation rate constants

Cholinesterase inhibited by Sevin is slowly reactivated by water. Thus a comparison of inhibition of cholinesterases by Sevin requires the determination of the inactivation rate constants under steady-state conditions.[10, 11] The inactivation rate constants were determined as described above. For bees, constant degrees of inhibition were obtained about 45 min after addition of low concentrations of Sevin to dilute brei. The constant inhibition continued for several hours. For flies, the steady-state condition was attained in less than 30 min. The cholinesterase of the diluted fly-brei was rather unstable, and the determinations had to be made within 1·5 hr. Figure 1 shows typical results of degrees of inhibition at various concentrations of Sevin. The bee

TABLE 1. INACTIVATION RATE CONSTANTS AND REACTIVATION RATE CONSTANTS FOR CHOLINESTERASES TREATED WITH SEVIN

Brei	$(I_{50})_{ss}$*	k'_3† Inactivation rate constant	k_4‡ Reactivation rate constant
Bee	$6{\cdot}7 \pm 0{\cdot}1 \times 10^{-9}$ M	$4{\cdot}0 \times 10^6$ M^{-1} min^{-1}	$0{\cdot}027 \pm 0{\cdot}003$ min^{-1}
Fly	$3{\cdot}9 \pm 0{\cdot}5 \times 10^{-8}$	$7{\cdot}4 \times 10^5$	$0{\cdot}029 \pm 0{\cdot}004$

* Average Sevin concentrations ± S.D. for 50 per cent inhibition at steady state.
† $k'_3 = k_4/(I_{50})_{ss}$.
‡ Average reactivation rate constants ± S.D.

data are averages of measurements made between 60 and 120 min of incubation, and the fly data between 30 and 75 min. The linear increase in inhibition ratio with increasing concentration of Sevin satisfies the steady-state equation.§ In Table 1 are given the concentrations of Sevin required, at steady state, for 50 per cent inhibition,

i.e. when $(I) = (I_{50})_{ss}$, or $(E'/E)_{ss} = 1$. The $(I_{50})_{ss}$ results shown in Table 1 are averages of several experiments. From $(I_{50})_{ss}$ and k_4 (determined below) the inactivation rate constant k'_3 can be calculated: $k'_3 = k_4/(I_{50})_{ss}$.

From Table 1 it can be seen that the inactivation rate constant for bee brei is over five times greater than that for fly brei.

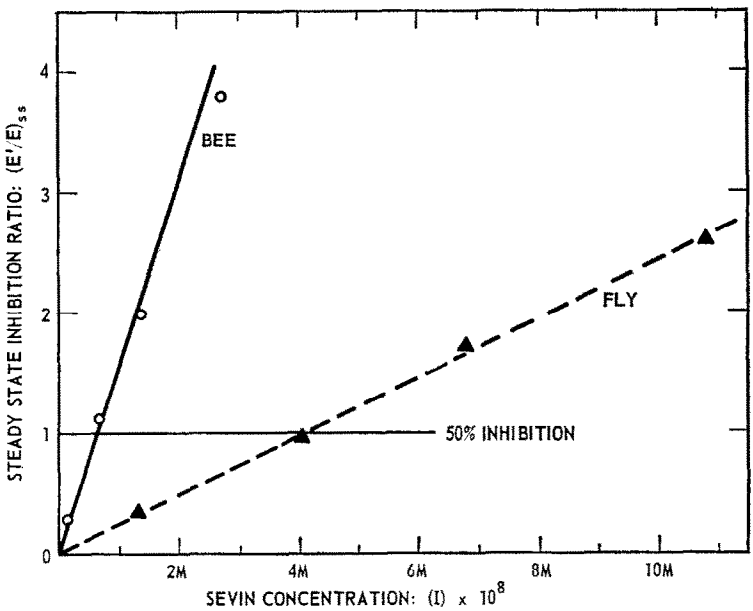


FIG. 1. Inactivation of cholinesterases by Sevin. Dilute breis of insect heads were incubated with the indicated concentrations of Sevin at 30°. After various time periods, samples were removed and assayed for cholinesterase activity. The data represent inhibition ratios measured after a constant degree of inhibition was obtained. The linear increase in inhibition with increase in Sevin concentration (I) satisfies the steady-state equation: $(E'/E) = (k'_3/k_4)(I)$.

Reactivation rate constants

The carbamyl–cholinesterase complex resulting from inhibition of cholinesterase by Sevin is slowly hydrolyzed, bringing about a reactivation of the enzyme.§ Reactivation rate constants were determined on breis which had been treated with Sevin to give about 75 per cent inhibition and then had been greatly diluted (see Materials and Methods). Figure 2 shows a typical decrease in fractional inhibition (E'/E'_0) of cholinesterase activity with time, t, from which the reactivation rate constant k_4 is calculated: $\ln (E'/E'_0) = -k_4 t$. The experimental technique was probably not precise enough for absolute evaluation of k_4; e.g. the reactivation occurring during the assay incubation time (5 min) was not negligible, yet a comparison of the constants of the two species could be made. The averages of reactivation rate constants determined from several experiments are given in Table 1. The difference in the average reactivation rate constants for the two species appears to be statistically insignificant.

DISCUSSION

The *reactivation* rate constants for bee and fly cholinesterases inactivated by Sevin were found to be very similar. However, the *inactivation* rate constants of the enzymes

were markedly different for the two species. The actual rates, *in vivo*, of inactivation (and reactivation) are, of course, dependent on such conditions prevailing in the tissue as substrate concentration, water concentration, pH, and the presence of reactivating material other than water. Because of the unknown factors affecting the *in vivo* conditions of the enzymes, factors which include membrane permeability to and penetration of inhibitor and other compounds, one cannot assume the conditions

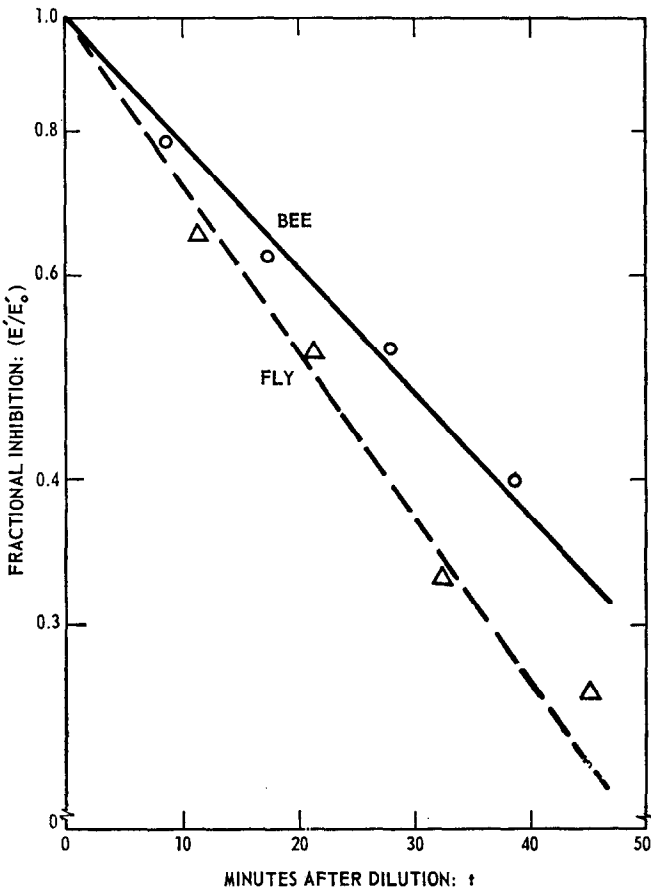


FIG. 2. Reactivation of cholinesterases inhibited by Sevin. Cholinesterase activity of insect-head breis was inhibited by Sevin and then greatly diluted to bring about reactivation. The diluted samples were assayed after various time periods for cholinesterase activity. (E'_0) is the concentration of inhibited enzyme at time of dilution and (E') is the concentration of inhibited enzyme at time after dilution; $k_4 = -\ln (E'/E'_0)/t$

of the cholinesterases, *in vivo*, to be the same for the two insects. But if one supposes these conditions are the same, then the difference between the inactivation rate *constants* found here would be reflected in a difference in the rates of inactivation of the cholinesterases. And the difference in the rates of inactivation of the enzymes by Sevin could explain, in part, the difference in sensitivity of honeybees and houseflies to Sevin.

It should be emphasized that insect breis were used rather than purified preparations in an effort to obtain rate constants representing all the cholinesterases present. Thus the values of k'_3 and k_4 found were dependent on both kinds (e.g. true acetylcholinesterase and pseudocholinesterase) and amounts of cholinesterases present in the mixtures. Satisfaction of the steady-state equation does not indicate the number of kinds of enzymes in the reaction mixtures but does indicate that all the cholinesterases being measured were stable during the period of inhibition. Satisfaction of the pseudo first-order equation indicates that the major cholinesterases present have approximately the same reactivation rate constants (an equal mixture of two cholinesterases with different reactivating rate constants would not give a logarithmic decrease in fractional inhibition with time during reactivation).

It would be of interest to compare the values of k'_3 and k_4 for mixtures of cholinesterases found in these insect breis with those found by Wilson *et al.* for purified electric eel acetylcholinesterase.[10] However, Sevin was not included in the monomethylcarbamates they examined; their measurements were made at pH 7, rather than pH 8 (optimal); and we used acetylthiocholine as a substrate, instead of acetylcholine. Acetylthiocholine was used for the measurements given here because of the ease of assay with this compound. Other work has indicated that this close analogue of the natural substrate is a satisfactory substitute for it,[8, 14] especially since relative values of rate constants were being determined.

Attempts have previously been made to correlate *in vivo* toxicity of organophosphates with sensitivity of brei cholinesterases from various species. Recently Dauterman and O'Brien[16] have repeated some of this work,[17, 18] using organophosphates of much higher purity than those used earlier. For these experiments the 50% inactivation concentration was used as index of sensitivity of cholinesterase. (Inactivation and reactivation rate constants cannot be measured as above with the organophosphates because the inactivated [phosphorylated] enzyme is stable.) The correlation between toxicity and sensitivity of bees and flies with the diisopropyl derivatives of parathion and paraoxon was striking, but was variable with the other phosphates tested.

Acknowledgement—The authors wish to thank Mr. Lee Watkins of the University Apiary for supplying the bees and Dr. Wendell Kilgore of this department for the flies.

This work was supported by United States Public Health Service Grant EF-00183.

REFERENCES

1. W. M. HOSKINS and S. NAGASAWA, *Botyu-Kagaku (Scientific Insect Control)* **26**, 115 (1961).
2. G. P. GEORGHIOU and R. L. METCALF, *J. econ. Entomol.* **55**, 125 (1962).
3. E. L. ATKINS, JR. and L. D. ANDERSON, *Proceedings of Conference on the Use of Agricultural Chemicals in California.* University of California, Davis (1963).
4. H. H. MOOREFIELD, *Misc. Publs. entomol. Soc. Amer.* **2**, 145 (1960).
5. M. J. KOLBEZEN, R. L. METCALF and T. R. FUKUTO, *J. agric. Food Chem.* **2**, 864 (1954).
6. D. K. MYERS, *Biochem. J.* **62**, 556 (1956).
7. W. C. DAUTERMAN, A. TALENS and K. VAN ASPEREN, *J. Insect Physiol.* **8**, 1 (1962).
8. R. E. KUNKEE and G. ZWEIG, *J. Insect Physiol.* **9**, 495 (1963).
9. I. B. WILSON, M. A. HATCH and S. GINSBURG, *J. biol. Chem.* **235**, 2312 (1960).
10. I. B. WILSON, M. A. HARRISON and S. GINSBURG, *J. biol. Chem.* **236**, 1498 (1961).
11. I. B. WILSON and M. A. HARRISON, *J. biol. Chem.* **236**, 2292 (1961).
12. R. L. METCALF and R. B. MARCH, *J. econ. Entomol.* **43**, 670 (1950).
13. W. W. KILGORE and R. R. PAINTER, *J. econ. Entomol.* **55**, 710 (1962).

14. G. L. ELLMAN, K. D. COURTNEY, W. ANDRES and R. M. FEATHERSTONE, *Biochem. Pharmacol.* **7**, 88 (1961).
15. I. B. WILSON in *The Enzymes*, P. D. BOYER, H. LARDY and K. MYRBÄCK, Eds., vol. 4, p. 501. Academic Press, New York (1960).
16. W. D. DAUTERMAN and R. D. O'BRIEN, *J. agric. Food Chem.* **12**, 318 (1964).
17. K. VAN ASPEREN and H. M. DEKHUIJZEN, *Biochim. biophys. Acta* **28**, 603 (1958).
18. R. L. METCALF and R. B. MARCH, *J. econ. Entomol.* **42**, 721 (1949).