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MALATHION POTENTIATION AND INHIBITION OF HYDROLYSIS OF VARIOUS CARBOXYLIC ESTERS BY TRIORTHOTOLYL PHOSPHATE (TOTP) IN MICE*†

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Abstract—Dose and time response relationships between triorthotolyl phosphate's (TOTP's) inhibition of liver carboxylesterase activity and its potentiation of malathion's anticholinesterase action in mice were studied. Both dose and time related conditions were discovered in which liver hydrolysis of diethyl succinate was completely inhibited by TOTP, but maximal potentiation of malathion was not observed under the same condition. Triacetin and methyl butyrate were included as carboxylesterase substrates in succeeding experiments. Considering these 3 substrates, TOTP's inhibition of liver triacetin hydrolysis was found to be most closely associated with the limiting conditions of TOTP pretreatment dose and time which resulted in potentiation of malathion's anticholinesterase action. Additional dose and time response studies showed that the degree of inhibition of hydrolysis of triacetin by livers from TOTP-pretreated mice was closely associated with both the degree of inhibition of liver hydrolysis of malathion and the degree of potentiation of malathion's anticholinesterase action *in vivo*. These experiments indicate that inhibition of liver hydrolysis of triacetin is a good indicator of conditions under which TOTP will potentiate malathion. Although inhibition of diethyl succinate and methyl butyrate hydrolysis was less specific for indicating conditions which result in malathion potentiation by TOTP, hydrolysis of these substrates was more sensitive than triacetin hydrolysis to inhibition by low doses of TOTP.

THE TOXICITY of the insecticide malathion [*O,O*-dimethyl-S-(1,2-dicarbethoxethyl) phosphorodithioate] is potentiated by several other insecticidal and noninsecticidal organic phosphates.[1–5] This has been attributed to inhibition of the enzymatic detoxification of malathion by the potentiating compounds.[6–9] The major enzymatic pathway for malathion detoxification in mammalian species is the hydrolysis of one of the carboxyester groups.[7,9,10] Recently, Chen *et al.*[11] showed that the enzymatic hydrolysis of malathion by rat liver occurs at the alpha carboxyester group. The enzymes that catalyze this reaction have been thought to belong to the carboxylic-ester hydrolase (I.U.B. No. 3.1.1.1, carboxylesterase) class of enzymes and have been referred to as aliesterases[9,12,13] or carboxyesterases.[14,15]

Acute toxicity studies of several combinations of organophosphate insecticides showed that the toxicity of malathion was potentiated more frequently than that of any other organic phosphate tested.[2] DuBois[13] and Murphy[16] have suggested that a

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carboxylesterase assay would be a useful method for screening compounds for possible potentiation of malathion or other compounds that are detoxified by carboxylesterases. Ideally, the substrate used for such assays would be the compound which might be potentiated. In the case of malathion potentiation, this presents difficulties because of limited substrate solubility and the presence of carboxylesterase-inhibiting contaminants or metabolites of malathion.[17,18] This has prompted the use of other carboxyesters as model substrates for studies of the relationships between carboxylesterase inhibition and potentiation among organophosphates.[9,12,15,19] These have included diethyl succinate (which chemically resembles the dicarbethoxy ethyl portion of malathion), triacetin, tributyrin, ethyl butyrate, and methyl butyrate, as well as malathion itself. All of these studies have supported the thesis that compounds that are potent carboxylesterase inhibitors are also potentiators of malathion's toxicity. However, little has been done to determine the relative value of various substrates used in carboxylesterase inhibition tests for predicting malathion potentiation.

In the present investigation three aliphatic esters, diethyl succinate (DES), triacetin (TA), and methyl butyrate (MeB), were used as carboxylesterase substrates to determine if they were equally valuable in predicting potentiation of malathion's anticholinesterase action *in vivo* in mice by triorthotolyl phosphate (TOTP), a noninsecticidal malathion synergist[3] and selective carboxylesterase inhibitor.[20]

MATERIALS AND METHODS

Male Swiss–Webster mice (Gofmoor Farms, 20–30 g) were used. They were housed in air-conditioned rooms (75–80°F) and were supplied with food and water *ad lib.* Corn oil solutions of malathion (analytical standard 98·5 per cent pure) and TOTP (practical grade) were prepared to provide the appropriate dose in an injection volume of 5 ml/kg. All injections were given intraperitoneally. Control animals received only corn oil. Animals were sacrificed by decapitation and exsanguination. Whole brains were homogenized in calcium-free Ringer–bicarbonate buffer. Livers were homogenized in 0·026 M sodium bicarbonate buffer, pH 7·6. Tissue homogenates were kept at 0–4° until the time of assay. All assays were completed within 5 days. Assays on tissues from control and TOTP-treated mice after various periods of storage showed that there was no significant change of activity during this time.

Brain cholinesterase activity was determined by the manometric method of DuBois and Mangun[21] with acetylcholine chloride (0·01 M) as the substrate. Liver carboxylesterase assays were performed in duplicate in a manner similar to that described previously.[15–18] Liver homogenates and bicarbonate buffer were placed in the main compartment of Warburg flasks, and 0·5 ml of the substrate solutions was added to the side-arm. The total volume was 3·0 ml. The flasks were gassed with 95% N_2–5% CO_2 for 5–7 min and equilibrated at 37·5° for 7–10 min. The substrates were tipped and manometer readings were taken at 5-min intervals for at least 20 min. Tissue and substrate blanks were included in each assay and the quantities of CO_2 produced in the experimental flasks were corrected for endogenous production of CO_2 and that resulting from non-enzymatic hydrolysis of the substrates. A preliminary series of experiments in which tissue levels and substrate concentrations were varied were conducted with livers of control mice in order to determine the optimum conditions at which tissue concentration would be the rate-limiting factor in the car-

boxylesterase assays. The tissue levels and substrate concentrations selected for routine assays and the enzyme activities of tissues from several control mice are shown in Table 1. Since malathion (or a metabolite, or a contaminant) has been reported to inhibit carboxylesterase activity,[18] its use as a substrate in these experiments was limited to two studies for comparison with other ester substrates.

Inhibition of brain cholinesterase was used as an index of malathion's toxicity in control or TOTP-pretreated mice. Since malathion itself inhibits liver carboxylesterase activity *in vivo*,[15,18] it was not possible to test for TOTP's inhibition of carboxylesterase and its potentiation of malathion in the same animals. Therefore, in experiments in which both parameters were tested, groups of eight or more mice were pretreated with either TOTP or corn oil. At selected intervals after pretreatment, half of the animals in each group were sacrificed and their brains and livers removed for cholinesterase and carboxylesterase assays respectively. At the same times after pretreatment, the remaining animals in each pretreatment group were challenged with malathion. Brains were removed from these mice at sacrifice (2 hr after malathion) or at death (if this occurred first) and were assayed for cholinesterase activity.

TABLE 1. BRAIN CHOLINESTERASE AND LIVER CARBOXYLESTERASE ACTIVITIES OF CONTROL MICE

Tissue	Substrate	Initial substrate concn (M)	mg of tissue* per assay	Activity† (μl CO_2/mg/15 min)
Brain	Acetylcholine	0·01	50·0	3·2 ± 0·04
Liver	Malathion	0·0067	200·0	0·6 ± 0·08
Liver	Diethylsuccinate	0·0067	2·5	60·9 ± 3·8
Liver	Triacetin	0·027	5·0	46·7 ± 4·9
Liver	Methylbutyrate	0·027	2·5	86·8 ± 5·0

* Fresh weight of tissue.
† Mean ± S. E. of eight or more animals.

For ease of discussion carboxylesterase activities are referred to according to substrate name (e.g. diethyl succinate esterase, triacetin esterase). This should not be interpreted to imply substrate specificity. Results are expressed as per cent of the mean activities of tissues from several control (corn oil-pretreated, unchallenged) mice that were included in each series of experiments.

RESULTS

Potentiation of malathion and diethyl succinate esterase inhibition by TOTP. In a previous study[22] we measured brain cholinesterase inhibition in control and TOTP-pretreated mice at 2 hr after intraperitoneal injections of various doses of malathion. At 16–18 hr after 125 mg/kg of TOTP, 10, 25, and 50 mg/kg of malathion produced 16, 43, and 73 per cent inhibition respectively. No significant inhibition of brain cholinesterase was produced by TOTP alone or by 600 mg/kg (or less) of malathion in corn oil-pretreated mice. No attempt was made in that study to determine the limiting dose of TOTP which would potentiate malathion's toxicity. The present investigation was designed to study the dose- and time-response relationships

between TOTP's potentiation of malathion and its inhibition of liver carboxylesterase activity. Initially, only diethyl succinate was used as the carboxylesterase substrate.

For dose–response studies, mice were pretreated with doses of TOTP ranging from 1 to 125 mg/kg. Sixteen to 18 hr later half of the animals in each dosage group were sacrificed and liver diethyl succinate esterase activities were measured. The remaining mice were challenged with 50 mg/kg of malathion, sacrificed 2 hr later and brain cholinesterase activities were measured. Fifty mg/kg of malathion was chosen as a challenge dose because our previous work[22] had shown that it was the maximal nonlethal dose in the linear portion of the malathion–anticholinesterase dose–response curve in mice pretreated with 125 mg/kg of TOTP, and because it was far below the cholinesterase-inhibiting dose for control mice. It seemed, therefore, that this dose of malathion would be most sensitive for detecting the minimal dose of TOTP which would produce potentiation of malathion. These conditions should then be optimal for detecting a correlation between malathion potentiation and liver carboxylesterase inhibition if the latter were the limiting mechanism for potentiation. The results of the TOTP dose–response tests are shown in Fig. 1. The degree of inhibition of brain cholinesterase by 50 mg/kg of malathion was dependent upon the dose of TOTP between 10 and 25 mg/kg. In contrast, diethyl succinate esterase activity was maximally inhibited by TOTP throughout this dose range.

Time–response relationships between diethyl succinate esterase inhibition and malathion potentiation were studied similarly, except that a constant dose of 125 mg/kg of TOTP was used, and the mice were either sacrificed or challenged with 50 mg/kg of malathion at various intervals after TOTP. The results are shown in Fig. 2. Even though diethyl succinate esterase inhibition was nearly maximal for 5 days, potentiation of the anticholinesterase action of 50 mg/kg of malathion was greatly diminished by the second day after TOTP pretreatment, and was not detectable at 5 days after TOTP.

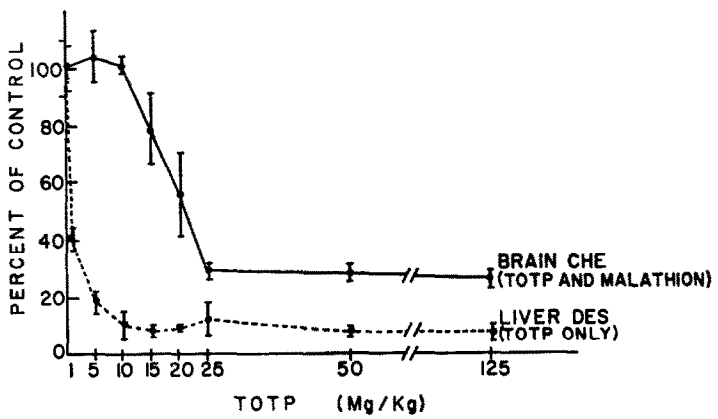

FIG. 1. Effect of triorthotolyl phosphate (TOTP) on mouse liver diethyl succinate (DES) esterase activity and on malathion's anticholinesterase action. Sixteen to 18 hr after TOTP, mice were either challenged with malathion (50 mg/kg) and sacrificed 2 hr later for brain cholinesterase (CHE) determination, or they were sacrificed without challenge for liver DES esterase determinations. Each point represents the mean ± S. E. of four or more mice. Values for corn oil controls were 3·2 ± 0·24 and 67·9 ± 6·8 μl CO_2/mg/15 min for brain CHE and liver DES esterase respectively.

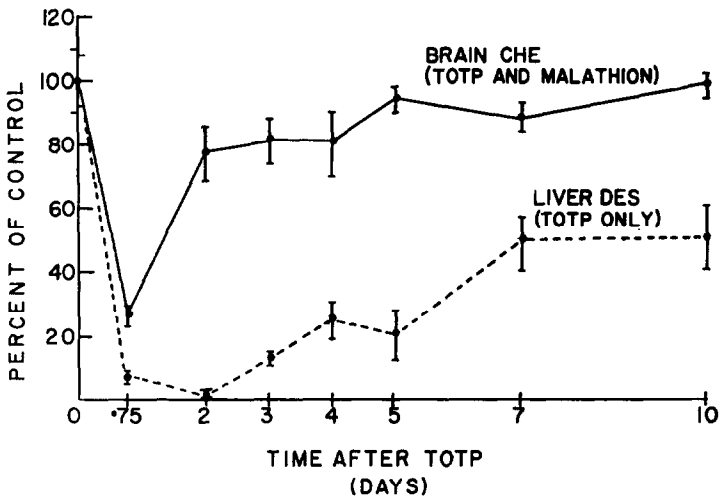

FIG. 2. Effect of triorthotolyl phosphate (TOTP) (125 mg/kg) on mouse liver diethyl succinate (DES) esterase activity and on malathion's anticholinesterase action. At the indicated times after TOTP, mice were either challenged with malathion (50 mg/kg) and sacrificed 2 hr later for brain cholinesterase (CHE) determinations, or they were sacrificed without challenge for liver DES esterase determinations. Each point represents the mean ± S.E. of four or more mice. Values for corn oil controls were 3·2 ± 0·24 and 67·9 ± 6·8 μl CO_2/mg/15 min for brain CHE and liver DES esterase respectively.

Comparison of inhibition of hydrolysis of various substrates by TOTP. The experiments described above indicated two situations in which diethyl succinate esterase activity was maximally inhibited by TOTP but malathion's toxicity was not potentiated. Because of this apparent lack of correlation between diethyl succinate esterase inhibition and malathion potentiation, the effect of TOTP on the capacity of mouse liver homogenates to hydrolyze other carboxyesters was investigated in order to determine if another substrate would be more useful for predicting TOTP's potentiation of malathion.

Groups of mice were pretreated with doses of TOTP between 1 and 25 mg/kg, sacrificed 16–18 hr later, and liver carboxylesterase activities measured with diethyl succinate, malathion, or triacetin as substrates. Results are shown in Fig. 3. Again the hydrolysis of diethyl succinate was found to be maximally inhibited between 10 to 25 mg/kg of TOTP. In contrast, the hydrolysis of both triacetin and malathion was dose–dependent within this dosage range. The dose–response curves for triacetin esterase and malathion esterase were nearly identical. The brain cholinesterase data from TOTP-pretreated, malathion-challenged mice (taken from Fig. 1) are plotted on the same graph for comparison. The dose–response curve for inhibition of brain cholinesterase by 50 mg/kg of malathion more nearly resembled the triacetin and malathion esterase inhibition curves than the diethyl succinate esterase inhibition curve.

The similarity in the dose–response curves for triacetin esterase and malathion esterase suggests that they may be identical enzymes or groups of enzymes. Furthermore, inhibition of triacetin esterase appeared to be a better indicator for predicting malathion potentiation than inhibition of diethyl succinate esterase. If this were true,

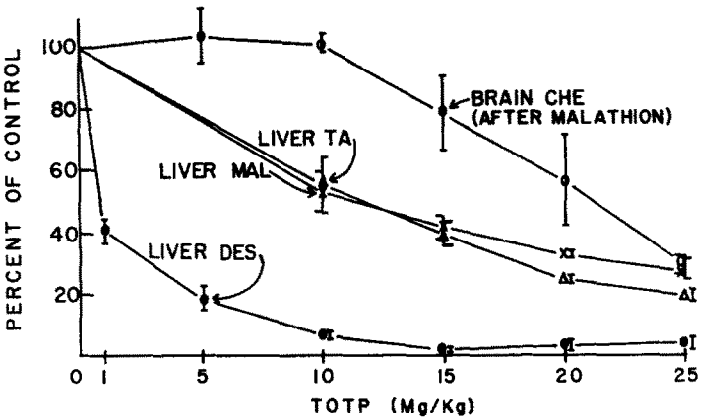

FIG. 3. Effect of triorthotoyl phosphate (TOTP) on mouse liver esterase activity and on malathion's anticholinesterase action. Sixteen to 18 hr after TOTP, mice were either sacrificed and liver diethyl succinate (DES), triacetin (TA) and malathion (MAL) esterase activities were determined, or they were challenged with malathion (50 mg/kg) and sacrificed 2 hr later for brain cholinesterase (CHE) determinations. Each point represents the mean ± S. E. of four or more mice. Values for corn oil controls were 3·2 ± 0·24, 55·2 ± 5·6, 46·7 ± 4·9 and 12·2 ± 0·2 μl CO_2/mg/15 min for CHE, DES, TA and MAL esterases respectively. (In a later experiment, liver TA and DES esterase activities at 18 hr after 5 mg/kg of TOTP were found to be 79 ± 6·8% and 15 ± 3·8% respectively.)

inhibition of triacetin esterase should also correlate more closely with malathion esterase inhibition and with potentiation of malathion's toxicity in time–response studies. The following experiments were designed to test this hypothesis. Groups of mice were injected with 125 mg/kg of TOTP and were sacrificed at 0·5, 1, 2, 2·5 and 5 hr later. Liver carboxylesterase activity was measured with diethyl succinate, malathion, triacetin, and methyl butyrate as substrates. The onset of inhibition of the hydrolysis of these substrates after TOTP is shown in Fig. 4. The rate of inhibition

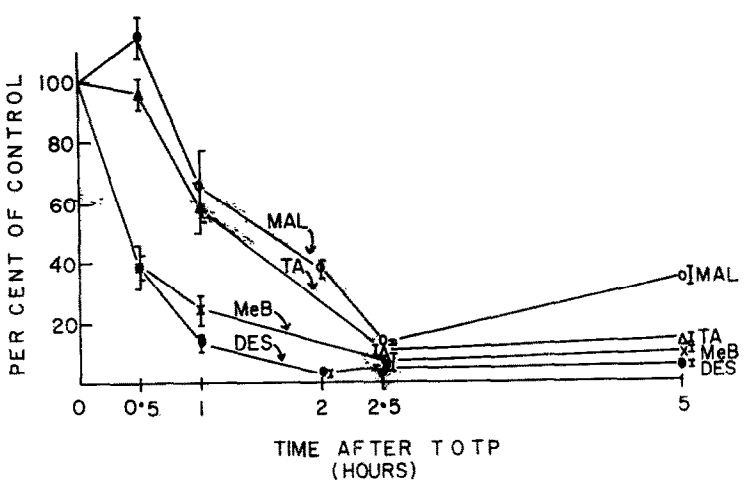

FIG. 4. Early effect of triorthotolyl phosphate (TOTP) (125 mg/kg) on mouse liver esterases. Mice were sacrificed at the indicated times after TOTP and liver hydrolysis of diethyl succinate (DES), triacetin (TA), methyl butyrate (MeB) and malathion (MAL) were determined. Each point represents the mean ± S.E. of four or more animals. Values for corn oil controls were 60·9 ± 3·8, 46·7 ± 4·9, 86·8 ± 5·0 and 12·2 ± 0·2 μl CO_2/mg/15 min for DES, TA, MeB and MAL esterases respectively.

was more rapid for diethyl succinate and methyl butyrate esterases than it was for malathion and triacetin esterases. Hydrolysis of all four ester substrates by mouse liver homogenates was maximally inhibited at 2·5 hr after TOTP administration. Thus, the onset of TOTP's effect on malathion esterase also correlated more closely with its effect on triacetin esterase than with its effect on either diethyl succinate or methyl butyrate esterases.

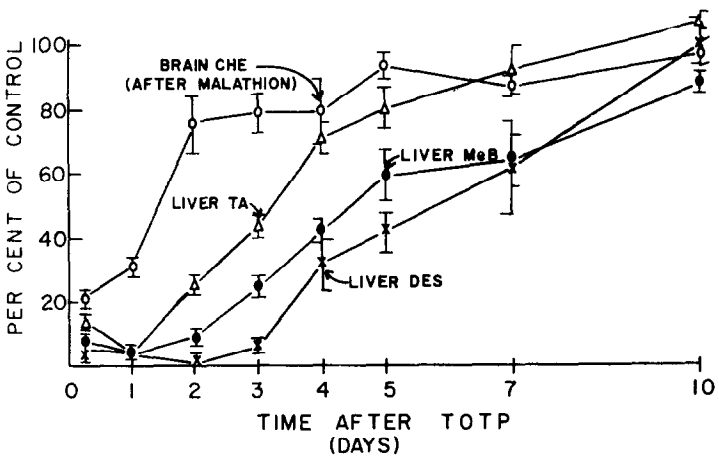

FIG. 5. Duration of triorthotolyl phosphate's (TOTP's) effect on liver esterase activities and on malathion's anticholinesterase action. At the indicated times after 125 mg/kg of TOTP, mice were either challenged with 50 mg/kg of malathion and sacrificed 2 hr later for brain cholinesterase (CHE) determinations or were sacrificed without challenge and liver carboxylesterase activities were measured. Diethyl succinate (DES), triacetin (TA) and methyl butyrate (MeB) were used as substrates in the carboxylesterase assays. Each point represents the mean ± S. E. of four or more animals. Values for corn oil controls were 3·2 ± 0·24, 60·9 ± 3·8, 46·7 ± 4·9 and 86·8 ± 5·0 μl CO_2/mg/15 min for CHE, DES, TA and MeB esterases respectively.

As a sequel to this experiment, the recovery patterns of liver esterases after TOTP treatment were examined. Groups of mice were injected with 125 mg/kg of TOTP and were sacrificed at 5 hr and at 1, 2, 3, 4, 5, 7 and 10 days later. Livers were assayed for triacetin, diethyl succinate, and methyl butyrate esterase activities. The results are shown in Fig. 5. At 10 days after TOTP pretreatment, liver hydrolysis of all three substrates had returned almost completely to control levels. Triacetin esterase activity recovered more rapidly than either diethyl succinate or methyl butyrate esterase activities and, of the three esterases studied, it was the only one to show statistically significant ($P < 0·05$) recovery by the second day after TOTP treatment. This recovery of triacetin esterase activity corresponded to the decrease in potentiation of the anticholinesterase action of the challenge dose of malathion (50 mg/kg) at 2 days after TOTP pretreatment.

Association between carboxylesterase inhibition and degree of malathion potentiation. The results of the preceding experiments indicated that inhibition of triacetin esterase accurately predicts the limiting conditions of TOTP pretreatment dosage and time which result in potentiation of malathion's anticholinesterase action. To be of maximum usefulness as a predictive assay, the degree of triacetin esterase inhibition should be quantitatively related to the degree of malathion potentiation. To test this,

the anticholinesterase action of various doses of malathion was measured in corn oil-pretreated mice and in mice at various intervals after pretreatment with 125 mg/kg of TOTP. The test intervals were chosen to correspond with points on the carboxylesterase recovery curves shown in Fig. 5. The results of the potentiation experiments are shown in Fig. 6.

There was no significant difference ($P > 0{\cdot}05$) in the brain cholinesterase inhibition curves between corn oil-pretreated malathion-challenged mice and mice challenged

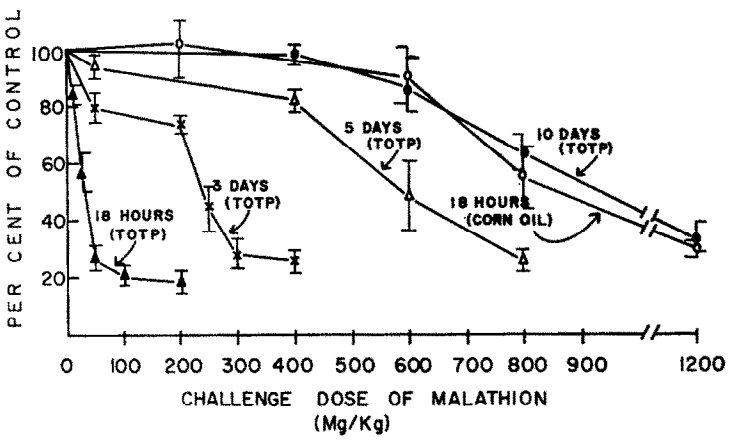

FIG. 6. Malathion's effect on brain cholinesterase activity at various times after 125 mg/kg of triorthotolyl phosphate (TOTP). Mice were challenged with malathion at the indicated times after TOTP and were sacrificed 2 hr later and brain cholinesterase activity was measured. Each point represents the mean ± S. E. of four or more animals. Control brain CHE activity was 3·2 ± 0·24 μl CO_2/mg/15 min.

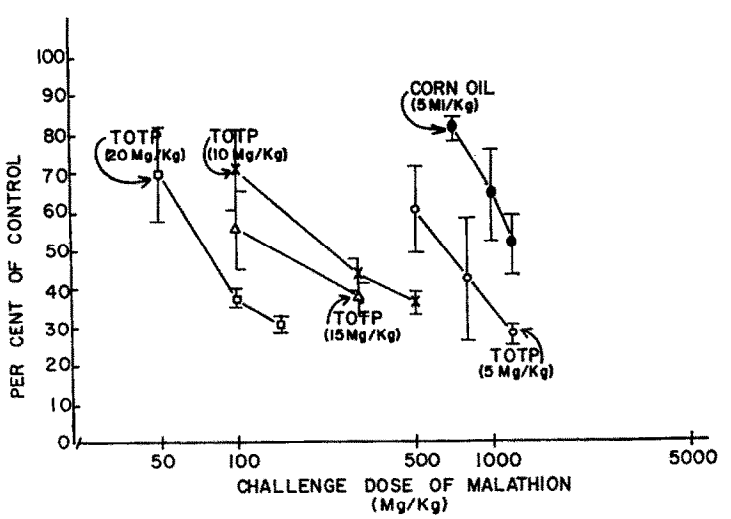

FIG. 7. Effect of malathion on brain cholinesterase activity after various doses of triorthotolyl phosphate (TOTP). Mice were challenged with malathion 16–18 hr after the indicated pretreatment dose of TOTP and were sacrificed 2 hr later and brain cholinesterase activity was determined. Each point represents the mean ± S. E. of four or more mice. Control brain CHE activity was 3·9 ± 0·09 μl CO_2/mg/15 min.

with malathion at 10 days after TOTP. This lack of potentiation was associated with the nearly complete recovery of liver carboxylesterase activity that was observed in unchallenged animals which were sacrificed 10 days after TOTP pretreatment (Fig. 5).

To assess the quantitative relationships between carboxylesterase inhibition and malathion potentiation at various times after TOTP, the cholinesterase ID_{50} for malathion (the dose which inhibited brain cholinesterase activity by 50 per cent) was determined from each of the curves in Fig. 6. From these values the degree of potentiation associated with each pretreatment interval was calculated (Table 2). Correlation

TABLE 2. LIVER ESTERASE INHIBITION AND DEGREE OF POTENTIATION OF MALATHION AT VARIOUS TIME INTERVALS AFTER 125 mg/kg OF TRIORTHOTOLYL PHOSPHATE (TOTP)

Time*	Malathion† brain CHE-ID_{50} (mg/kg)	Degree of potentiation‡	Per cent inhibition of liver hydrolysis after TOTP§		
			DES	TA	MeB
18 hr	30	28·6	96	96	95
3 days	240	3·6	93	56	75
5 days	590	1·4	57	20	40
10 days	935	0·9	0	0	11
Control‖	860				

Correlation coefficients—Per cent inhibition of liver esterases vs. degree of potentiation
DES (r = 0·602); TA (r = 0·879); MeB (r = 0·669)

* Time interval between pretreatment with TOTP and challenge with malathion.

† Malathion dose (mg/kg) causing 50 per cent inhibition of brain cholinesterase, determined from Fig. 6.

‡ Degree of potentiation = ID_{50} (control)/ID_{50} (TOTP pretreated).

§ Values for corn oil controls were 60·9 ± 3·8, 46·7 ± 4·9 and 86·8 ± 5·0 μl CO_2/mg/15 min for DES, TA and MeB esterases respectively.

‖ Control mice were pretreated with corn oil 18 hr prior to challenge with malathion.

coefficients (r) for the association between degree of potentiation and per cent inhibition of liver carboxylesterase activities in unchallenged TOTP-pretreated mice at corresponding time intervals (Fig. 5) were calculated.[23] These are summarized in Table 2. Inhibition of liver triacetin esterase activity was most closely associated with the degree of malathion potentiation in mice challenged at selected time intervals after TOTP pretreatment. This relationship was further tested by determining the anticholinesterase action of selected doses of malathion in mice which had been pretreated 16–18 hr earlier with 5, 10, 15 or 20 mg/kg of TOTP (Fig. 7). Brain cholinesterase ID_{50}'s for malathion were determined from each of the curves in Fig. 7, and the degree of malathion potentiation was calculated for each TOTP pretreatment dose (Table 3). Correlation coefficients were calculated for the association between degree of potentiation and per cent inhibition of liver carboxylesterase activities at corresponding TOTP dosages (determined from Fig. 3). Triacetin and malathion esterase inhibition were both more closely associated than diethyl succinate esterase inhibition with the degree of malathion potentiation observed after the different doses of TOTP.

TABLE 3. LIVER ESTERASE INHIBITION AND DEGREE OF POTENTIATION OF MALATHION 16–18 hr AFTER SELECTED DOSES OF TRIORTHOTOLYL PHOSPHATE (TOTP)

Pretreatment dose of TOTP (mg/kg)	Malathion* brain CHE-ID_{50} (mg/kg)	Degree of† potentiation	Per cent inhibition of liver hydrolysis after TOTP‡		
			DES	TA	MAL
5	660	1·9	84	21	22
10	230	5·4	93	45	47
15	145	8·6	98	60	59
20	76	16·4	97	75	67
Control§	1250		0	0	0

Correlation coefficients—Per cent inhibition of liver esterases vs. degree of potentiation
DES ($r = 0·705$); TA ($r = 0·951$); MAL ($r = 0·895$)

* Malathion dose (mg/kg) causing 50 per cent inhibition of brain cholinesterase, determined from Fig. 7.

† Degree of potentiation $= \frac{ID_{50} \text{ (corn oil pretreatment)}}{ID_{50} \text{ (TOTP pretreatment)}}$.

‡ Values for corn oil controls were 67·6 ± 4·1, 42·8 ± 3·1 and 14·3 ± 0·07 μl CO_2/mg/15 min for DES, TA and MAL esterases respectively.

§ Control mice were pretreated with corn oil 18 hr prior to challenge with malathion.

DISCUSSION

DuBois[13] and Murphy[16] suggested that inhibition of tissue carboxylesterase activity would be useful for detecting chemicals that will potentiate the toxicity of malathion or other compounds that are detoxified by carboxylesterases. If a carboxylesterase assay is to be of maximum usefulness for this purpose, it should not only be sensitive to inhibition by potentiators but it should also be sufficiently specific to differentiate between weak and strong potentiators and to reveal the temporal and dosage conditions which result in potentiation *in vivo*. The initial experiments in this investigation showed that the degree of inhibition of diethyl succinate hydrolysis by mouse liver after various doses and at various time intervals after TOTP did not correspond closely to the degree of potentiation of malathion's anticholinesterase action *in vivo*.

Additional dose and time–response studies showed that the degree of inhibition of hydrolysis of triacetin by livers from TOTP-pretreated mice was more closely associated with the degree of potentiation of malathion's anticholinesterase action by TOTP *in vivo* than was the degree of inhibition of either diethyl succinate or methyl butyrate hydrolysis.

Inhibition of liver triacetin esterase activity after varying doses or at various times after TOTP was also more closely associated with inhibition of malathion's hydrolysis than was inhibition of either diethyl succinate or methyl butyrate esterase activities. Thus, these experiments indicate that triacetin is a useful model substrate for measuring TOTP's inhibition of malathion detoxification.

Although triacetin esterase inhibition was more specific for indicating critical time and dosage variables which result in potentiation of malathion's toxicity in mice by TOTP, triacetin esterase was not as sensitive as diethyl succinate esterase to inhibition by TOTP. Ten mg/kg of TOTP was required to produce approximately 50 per cent

inhibition of mouse liver triacetin esterase while the same degree of inhibition of diethyl succinate esterase was produced by only 1 mg/kg of TOTP.

Casida[4] compared the potentiation of malathion in mice with the inhibition of malathion hydrolysis by liver and plasma at 24 hr after a standard dose (100 mg/kg) of 43 different aryl phosphorus compounds. He found that the activities of the compounds in producing inhibition of the malathion-hydrolyzing esterases *in vivo* were generally related to their ability to increase susceptibility of the mice to poisoning by malathion. The correlation between malathion potentiation and malathion esterase inhibition was better with liver esterase than with plasma. Casida's data show that compounds that produced an average of 46 per cent inhibition of liver malathion esterase activity potentiated malathion's acute toxicity (compared by calculating ratios of malathion's LD_{50} in his control mice/LD_{50} in his pretreated mice) by 1·7- to 3·0-fold. The same degree of inhibition of liver hydrolysis of triacetin (45 per cent) and malathion (47 per cent) was produced by 10 mg/kg of TOTP in this investigation (Table 3), and we found a corresponding 5·4-fold potentiation of the anticholinesterase action of malathion *in vivo*. In Casida's study, six compounds produced an average of 65 per cent inhibition of liver hydrolysis of malathion and they potentiated malathion's acute toxicity (LD_{50}'s) by 4·7- to 7·5-fold. In our study (Table 3), comparable degrees of inhibition of malathion hydrolysis (67 per cent) and triacetin hydrolysis (75 per cent) were produced by 20 mg/kg of TOTP which potentiated malathion's anticholinesterase action *in vivo* by 16·4-fold. Thus, Casida's and our results are comparable in terms of demonstrating a quantitative correlation between malathion hydrolysis and potentiation of malathion in mice. Additionally, we have shown that, at least for TOTP, triacetin can be used as a substitute substrate for malathion in comparing liver esterase inhibition with malathion potentiation in mice. For an equivalent degree of inhibition of liver malathion esterase, we found about twice the degree of potentiation of malathion by using brain cholinesterase inhibition as the index of toxicity compared to calculated values using Casida's LD_{50}'s. This suggests that ratios of control/pretreated ID_{50}'s for brain cholinesterase may be more sensitive for detecting potentiation than ratios of control/pretreated LD_{50}'s. However, other explanations could account for this, since recent experiments in progress in our laboratory indicate that the quantitative relationship between liver carboxylesterase inhibition and malathion potentiation observed in this study with TOTP may not hold for all potentiators of malathion.

DuBois[13] reported dietary concentrations that produced 50 per cent inhibition of hydrolysis of diethyl succinate and tributyrin in weanling female rats fed several organophosphorus compounds in the diet. We found in mice that liver hydrolysis of diethyl succinate was about 10 times as sensitive to inhibition by single intraperitoneal doses of TOTP as the liver hydrolysis of triacetin (a glyceryl triester). DuBois found in their feeding studies that diethyl succinate hydrolysis by liver was about three times as sensitive as tributyrin hydrolysis to inhibition by TOTP or Folex (tributyl phosphorotrithioate) in the diet. However, liver diethyl succinate hydrolysis was less sensitive than liver tributyrinase to inhibition by seven other compounds fed in the diet. Data from feeding studies in adult male rats obtained by Murphy and Cheever[15] indicate that liver hydrolysis of triacetin is slightly more sensitive than hydrolysis of diethyl succinate to inhibition by malathion, parathion and doxathion [2,3-P-dioxanedithiol S,S'-*bis* (*O,O*-diethyl phosphorodithioate)], but no difference was noted for

inhibition of hydrolysis of these two substrates by ronnel (*O,O*-dimethyl *O*-2,4,5-trichlorophenyl phosphorothioate).

The differences in sensitivity of triacetin (or tributyrin) esterase and diethyl succinate esterase to inhibition by organophosphate insecticides suggest that at least part of the activities for these two substrates are contributed by different enzymes or groups of enzymes. Such evidence to support this hypothesis was obtained in an experiment conducted during the present investigation. We measured the CO_2 produced when saturation concentrations of both diethyl succinate and triacetin were incubated with 2·5 mg of mouse liver and compared this with the amount of CO_2 produced with each substrate alone. If the same enzyme catalyzed hydrolysis of both substrates, it would be expected that the CO_2 produced in the combined substrate incubate would not be appreciably different from that of each substrate alone. We found, however, that 197·0 μl of CO_2 was produced in the combined substrate incubate, 113·5 μl with diethyl succinate alone and 96·5 μl with triacetin alone. Thus, the activities were approximately additive which suggests that different enzymes acted upon the two substrates independently. Further studies are needed to confirm this, but Iwatsubo[24] has shown that liver esterase groups that are classified by organophosphate sensitivity can be further separated by electrophoresis.

Although the present study indicates that triacetin esterase inhibition is a good indicator of conditions under which TOTP will potentiate malathion, it would be premature to suggest that this substrate would be superior to other carboxyesters for predicting increased susceptibility to malathion produced by other potentiating compounds. Additional data of the type reported here are needed for other compounds before such a generalization can be made. This and other studies indicate that the carboxylic ester substrate whose hydrolysis is most sensitive to inhibition would provide the greatest margin of safety. Unfortunately, this may differ with different compounds and inhibition of the most sensitive carboxylesterase may not always correlate most closely with potentiation *in vivo*.

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