Biochemical Pharmacology, Vol. 26, pp. 1791–1795. Pergamon Press, 1977. Printed in Great Britain.

TRIORTHOTOLYL PHOSPHATE INHIBITION OF CARBOXYLESTERASES AND POTENTIATION OF PROCAINE TOXICITY*†

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(*Received* 1 *October* 1976; *accepted* 21 *January* 1977)

Abstract—Groups of mice were given single doses of triorthotolyl phosphate (TOTP) (1–125 mg/kg, i.p.) 18 hr prior to sacrifice or procaine challenge. A dose-dependent decrease was recorded in liver carboxylesterase hydrolysis of diethyl succinate, triacetin and procaine. TOTP pretreatment shortened the time to onset and prolonged the duration of procaine (175 mg/kg, i.p.)-induced loss of righting ability. The correlation coefficient for inhibition of liver procaine hydrolysis and prolongation of procaine action was 0.97 ($P < 0.0001$). No deaths were observed in procaine-injected controls yet mortality after procaine injection in TOTP-pretreated mice increased as a function of the TOTP pretreatment dose. In addition, plasma procaine concentrations after procaine injection (150 mg/kg, i.p.) reached peak levels sooner, remained elevated longer and were 3-fold higher in the TOTP-pretreated mice as compared to corn oil-pretreated procaine-injected controls. Results demonstrate potentiation of procaine toxicity by TOTP and suggest that this results from inhibition of procaine metabolism *in vivo*.

Potentiation of malathion [*O,O* dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate] toxicity by triorthotolyl phosphate (TOTP) and other organophosphates has been shown to result primarily from inhibition of tissue carboxylesterases (EC 3.1.1.1) which are responsible for malathion hydrolysis [1–3]. There have been many studies of toxicologic interactions between those organophosphates which inhibit carboxylesterases and those organophosphates, like malathion, whose major pathways of detoxification involve carboxylester hydrolysis [1–7]. In contrast, little has been done to study the effect of organophosphate inhibition of esterases on the metabolism and toxicity of ester drugs. Bis-(*p*-nitrophenyl) phosphate inhibited acetanilide hydrolysis and prevented acetanilide-induced methemoglobinemia [8]. Likewise EPN [*O*-ethyl, *O*-*p*-nitrophenyl, phenyl phosphonothioate] inhibited the hydrolysis of isocarboxazid and diminished isocarboxazid-induced inhibition of monoamine oxidase activity [9]. In both cases, drug hydrolysis was a prerequisite to drug action. More recently procaine metabolism was shown to be inhibited by EPN and enhanced by phenobarbital. EPN pretreatment retarded and phenobarbital pretreatment enhanced clearance of injected procaine from rat tissues [10]. The present study was undertaken to relate organophosphate inhibition of procaine hydrolysis with organophosphate potentiation of procaine toxicity in mice.

MATERIALS AND METHODS

Adult male Charles River mice (CD-1, 25–35 g) were used for this study. They were kept in air-conditioned (25°) animal quarters and given Purina Laboratory Chow and water *ad lib*. Mice were housed six/cage in stainless steel cages. The cages had a grid flooring which was suspended 2.5 inches above waste pans containing sawdust. Animal quarters were on a 12 hr (7–7) light–dark cycle. All injections were timed to permit sacrifice or challenge between 8:00 and 11:00 a.m. Procaine HCl (Merck) was dissolved in distilled water and TOTP (Eastman) was dissolved in corn oil to provide the appropriate dose in an injection volume of 5 ml/kg. All injections were made intraperitoneally. Control mice were given vehicle only. Mice were sacrificed by decapitation and exsanguination and tissues were homogenized in the appropriate buffer at 0–4°. Homogenates were either used the same day or were stored frozen and assayed within 3–4 days. There was no loss of enzyme activity under these conditions. The Student's *t*-test was used to compare experimental and control groups. Values of $P < 0.05$ were considered significant.

Determination of esterase activities. Tissue levels and initial concentrations of substrates are listed in Table 1 along with representative activities from control mice. Carboxylesterase activity was determined manometrically in sodium bicarbonate buffer (0.026 M, pH 7.6) at 37° with diethyl succinate and triacetin as substrates [11]. Liver hydrolysis of procaine in sodium phosphate buffer (0.067 M, pH 7.2) was determined by dual wavelength (290 and 265.5 nm) analysis.‡ Procaine HCl was incubated with homogenized liver for 1 hr at 37°. The incubation was stopped by addition of trichloroacetic acid (20%, w/v). The mixture was filtered, the filtrate diluted and the absorbance measured at the indicated

* Presented in part at the annual meeting of the Society of Toxicology, Atlanta, GA. March, 1976.

† Supported in part by a grant from the University of Connecticut Research Foundation and by NIH Research Grant ES 01093 O1A1 from the National Institute of Environmental Health Sciences.

‡ S. D. Cohen, M. Ehrich and D. Orzech, manuscript submitted for publication.

Table 1. Tissue esterase activities of control mice

Substrate* (M)	Tissue† (mg)	Activity‡ (μmoles/min/g)
Diethyl succinate (0.0067)	Liver (2.5)	193 ± 9
Triacetin (0.027)	Liver (5.0)	121 ± 12
Procaine (0.020)	Liver (100.0)	0.80 ± 0.1
Procaine (0.004)	Plasma (250.0)	0.005 ± 0.001
Acetyl thiocholine (0.001)	Brain (0.8)	12.3 ± 0.5
Acetyl thiocholine (0.001)	Liver (1.0)	8.9 ± 0.3
Acetyl thiocholine (0.001)	Plasma (10.0)	13.3 ± 0.4

* Initial molar concentration.
† Wet weight of tissue.
‡ Mean ± S. E. of five or more control mice.

wavelengths. Procaine concentration was determined using the molar absorptivities of procaine and its hydrolysis product, *para*-aminobenzoic acid, at both wavelengths. To determine mouse plasma hydrolysis of procaine, 200 μg procaine was added to 0.25 ml plasma in sufficient 0.1 M sodium borate buffer (pH 9.0) to make a final incubate volume of 2.0 ml. After incubation with shaking for 60 min at 37°, ethyl acetate (5 ml) was added and procaine extraction was accomplished by mixing (Vortex mixer) for 30 sec. The absorbance of the ethyl acetate layer was measured at 290 nm and compared to a standard curve to determine the amount of procaine remaining in the incubate. The standard curve was prepared by adding known amounts of procaine to control plasma in borate buffer and extracting immediately (without incubation) into ethyl acetate. The amount of procaine hydrolyzed was calculated as the difference between procaine remaining in the incubated samples and that remaining in a similarly treated procaine blank which was incubated without plasma. Tissue cholinesterase activities were determined by the spectrophotometric method of Ellman *et al.* [12] in 0.1 M sodium phosphate buffer (pH 8) at 27°. In all cases, tissue and substrate concentrations were selected to provide optimum conditions at which enzyme activity of the tissue would be rate limiting. All assays were performed in duplicate and were corrected for nonenzymatic hydrolysis of substrates and for tissue interference by using appropriate tissue and substrate blanks.

Determination of plasma procaine levels. At selected times after injection of procaine HCl (150 mg/kg), mice were decapitated and blood was collected in oxalate-rinsed (25% potassium oxalate) centrifuge tubes. To prevent procaine hydrolysis by plasma esterases, 25% NaF solution (2 drops) was added to the collected blood. Individual samples were kept on ice until all samples were collected, and then all were centrifuged at 1500 rev/min for 15 min. To 1.75 ml borate buffer (pH 9), 0.25 ml plasma was added and followed by 5 ml ethyl acetate. The tube contents were mixed for 30 sec (Vortex mixer) and then centrifuged at 1500 rev/min for 10 min. Absorbance of the ethyl acetate layer was determined at 290 nm. Preliminary tests involving repeated extractions indicated that this procedure removed 98 per cent of the added procaine. Correction was made for absorbance of tissue by extracting control plasma in the above manner. The amount of procaine in the samples was determined by comparing the absorbance of the sample extracts to a standard curve prepared by adding known amounts of procaine to 0.25 cc of NaF-treated control plasma and following the above procedure.

Determination of procaine toxicity. Procaine toxicity was measured by determining the time to and duration of loss of righting ability, after a sublethal dose of procaine (175 mg/kg). Onset was measured as the time elapsed from injection to loss of righting ability. Recovery was measured from the loss to the regaining of righting ability. A mouse was considered to have recovered if it could right itself after being turned on its back, twice in a 10-sec time period.

RESULTS

Effect of TOTP pretreatment on mouse liver and plasma esterase activity. To determine the effect of TOTP on the esterase activity of mouse liver, groups of five mice were pretreated with selected doses of TOTP or corn oil and sacrificed 18 hr later. Results are shown in Fig. 1. Inhibition of carboxylesterase activity was dependent on the dose of TOTP given. The degree of inhibition of ester hydrolysis after similar doses of TOTP varied among the individual substrates tested. Brain cholinesterase activity (not shown) was not significantly ($P > 0.05$) altered after any of the TOTP doses tested. For the ester substrates tested, liver hydrolysis of diethyl succinate was most sensitive to inhibition by low doses of TOTP. Twenty per cent inhibition was detected after only 1 mg TOTP/kg, whereas procaine and triacetin hydrolysis were not significantly affected by this dose. Maximal inhibition of diethyl succinate hydrolysis occurred after 10 mg TOTP/kg. Inhibition detected after higher TOTP doses was not significantly greater ($P > 0.05$) than that observed after 10 mg TOTP/kg. Procaine hydrolysis was maximally inhibited by 25 mg TOTP/kg.

To determine the effect of TOTP on plasma cholinesterase and plasma hydrolysis of procaine, additional groups of five mice were pretreated with similar doses of TOTP, and 18 hr later, they were sacrificed and the blood was collected and assayed immediately. Liver cholinesterase activity was also determined. Results are shown in Fig. 2. The effect of TOTP on liver hydrolysis of procaine has been replotted from Fig. 1 for comparison. Plasma cholinesterase activity was not inhibited by 5 mg TOTP/kg, whereas liver cholinesterase activity and

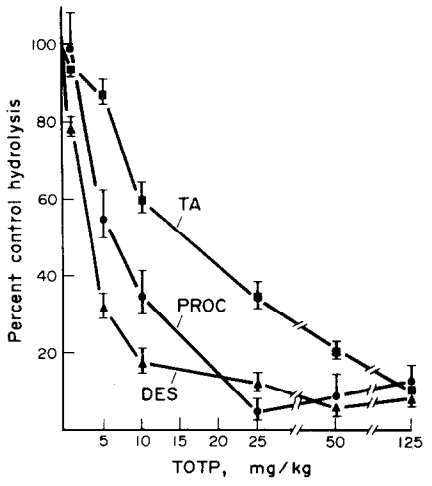

Fig. 1. Mouse liver hydrolysis of triacetin (TA), diethyl succinate (DES), and procaine (PROC) 18 hr after TOTP injection. Each point represents the mean ± S. E. of groups of five to ten mice.

both liver and plasma procaine hydrolysis were significantly inhibited by this dose. Furthermore, neither liver procaine hydrolysis nor plasma cholinesterase was inhibited by 1 mg TOTP/kg, whereas liver cholinesterase and plasma procaine hydrolysis were significantly inhibited by this low dose of TOTP. Inhibition of liver and plasma cholinesterase and liver procaine hydrolysis by 125 mg TOTP/kg was not significantly greater ($P > 0.05$) than that observed after 25 mg/kg. In contrast, maximal inhibition of plasma procaine hydrolysis occurred after 5–10 mg TOTP/kg and this amounted to only 45–55 per cent inhibition.

Effect of TOTP pretreatment on the toxicity of procaine in the mouse. The preceding results demonstrated that TOTP markedly inhibited liver hydrolysis of procaine at doses below those which inhibited brain cholinesterase. Thus, doses of TOTP which produced no symptoms of cholinergic poisoning might enhance procaine toxicity, if hydrolysis by liver were an important pathway for procaine detoxification in the mouse. To test this, groups of five or more mice were pretreated with selected doses of TOTP, and 18 hr later, they were challenged with 175 mg/kg of procaine HCl, i.p., for determination of procaine toxicity. Results are shown in Table 2. The dose of procaine (175 mg/kg) was selected, based on preliminary tests which demonstrated that it caused a measurable loss of righting ability without lethality in control mice.

In control mice, loss of righting ability occurred in 195 sec, and lasted 259 sec, after procaine injection. TOTP pretreatment shortened the time to onset, and significantly ($P < 0.05$) lengthened the duration of procaine action. Duration of procaine action increased as a function of the TOTP pretreatment dose. In addition, mortality after procaine (175 mg/kg) also increased as the TOTP dose increased. Just as 1 mg/kg had little or no effect on ester hydrolysis by mouse liver, this dose did not significantly alter recovery time. However, one of the mice in this group died after procaine treatment. Correlation coefficients for the association between duration of procaine action and liver and plasma esterase activities after TOTP were: −0.9875, −0.8888, −0.9887, −0.9717, −0.9157 and −0.9623 for liver cholinesterase and hydrolysis of diethyl succinate, triacetin and procaine, and plasma cholinesterase and hydrolysis of procaine, respectively. All were statistically significant ($P < 0.05$) and suggest a strong relationship between the inhibition by TOTP of esterases and its potentiation of procaine toxicity.

Effect of TOTP pretreatment on the time course of plasma procaine levels in mice. The above findings have demonstrated that TOTP pretreatment potentiated the toxicity of subsequently administered procaine. Furthermore, metabolism of procaine was inhibited in livers from TOTP-treated mice. To determine if a similar inhibition of procaine metabolism occurred when procaine was administered *in vivo*, the plasma procaine levels in both control and TOTP-pretreated mice were compared after injection of procaine. If potentiation occurred as a result of altered metabolism of procaine *in vivo* one would expect to see elevated plasma procaine levels in TOTP-treated mice. Groups of mice were pretreated with corn oil, or 25 mg TOTP/kg, and 18 hr later they were challenged with 150 mg procaine/kg, i.p. The mice were sacrificed at selected times from 1 to 60 min after procaine challenge, and the blood was collected for analysis of procaine content. Results are shown in Fig. 3. The peak procaine level reached in control mice was 30 μg/ml, whereas the peak level in the TOTP-treated mice was approximately 100 μg/ml. Peak level was attained by 3 min after injection in control mice. In contrast, in the TOTP-treated mice, the peak level appeared to have already been reached at 1 min (the earliest sampling time tested). Within 40 min after injection, procaine could be considered gone from the plasma of control mice. Yet up to 60 min after injection, the TOTP-treated mice showed procaine levels of approximately 10 μg/ml. Thus, it appears that the peak plasma level was reached sooner, rose approximately three times higher, and remained elevated longer in the TOTP-pretreated mice compared to controls. No deaths were observed

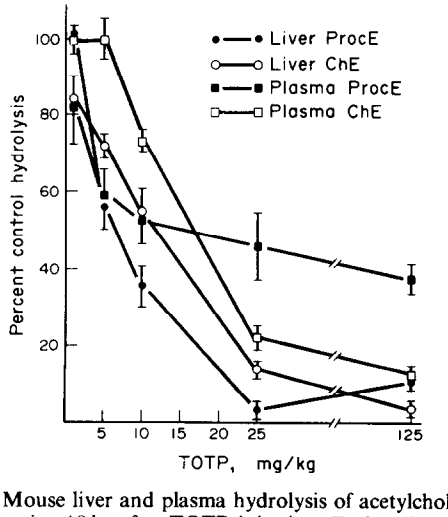

Fig. 2. Mouse liver and plasma hydrolysis of acetylcholine and procaine 18 hr after TOTP injection. Each point represents the mean ± S. E. of groups of five to ten mice.

Table 2. Effect of TOTP pretreatment on the toxicity of subsequently administered procaine*

TOTP (mg/kg)	Onset† (sec)	Recovery† (sec)	Number dead / Number injected
0	195 ± 11	259 ± 23	0/16
1	170 ± 8	304 ± 29	1/10
5	150 ± 13‡	634 ± 102§	1/5
10	176 ± 29	1017 ± 89§	2/5
25	154 ± 29‡	1519 ± 149§	6/10
125	142 ± 12§	1620, 1710‖	8/10

* TOTP was injected 18 hr prior to procaine (175 mg/kg) challenge. Control groups received corn oil instead of TOTP. Values in table represent mean ± S. E. For onset, N = number injected; for recovery, N = number alive.

† Onset is equal to the time from injection to loss of righting ability and recovery is equal to the time between the loss and the regaining of righting ability.

‡ Significantly different from control ($P < 0.05$).

§ Significantly different from control ($P < 0.01$).

‖ Individual recovery times for the two survivors.

in controls, whereas fourteen TOTP-treated mice died prior to their set sacrifice time. The mean ± S. E. plasma procaine concentration of these mice was 99.4 ± 4.5 μg/ml. The data for these mice were not included in the preparation of Fig. 3. Since the preceding experiments indicated that maximal inhibition of liver hydrolysis of procaine and maximal prolongation of procaine-induced loss of righting ability occurred after 25 mg TOTP/kg, one would predict that 125 mg TOTP/kg should have no additional effect on plasma procaine levels beyond that observed after 25 mg/kg. This was tested, and plasma procaine levels at 1 or 3 min after procaine in TOTP (125 mg/kg)-pretreated mice were the same ($P > 0.05$) as those detected in mice pretreated with 25 mg/kg.

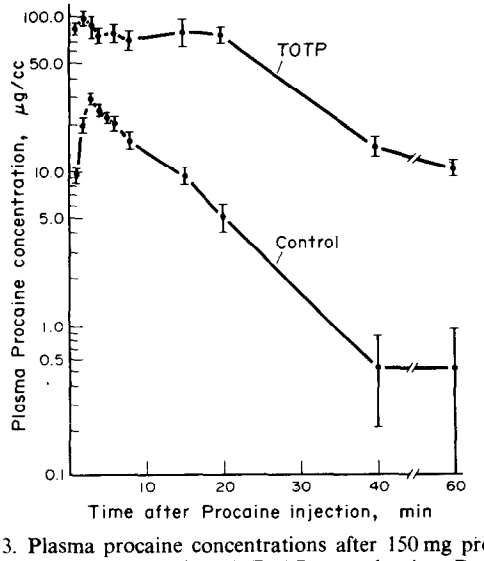

Fig. 3. Plasma procaine concentrations after 150 mg procaine/kg, i.p. in control and TOTP-treated mice. Each point represents the mean ± S. E. of concentrations in four to six mice. TOTP pretreatment (25 mg/kg) was given 18 hr prior to procaine.

DISCUSSION

This research provided a systematic study of the toxicologic interaction between TOTP and procaine. Liver and plasma taken from mice treated with carboxylesterase-inhibiting doses of TOTP had a decreased capacity to metabolize procaine, *in vitro*. Similar effects probably also occurred *in vivo*, since plasma procaine concentrations reached higher levels and remained elevated longer in procaine-injected mice which had been pretreated with TOTP than in procaine-injected controls. Furthermore, TOTP pretreatment enhanced the acute toxicity of procaine in mice. Recovery from procaine-induced loss of righting ability was delayed in TOTP-pretreated mice and several of the TOTP-treated mice died after challenge with an apparently non-lethal (in controls) dose of procaine. We felt it important to determine if an interaction might occur under conditions in which the test animal was not overloaded with ester drug. Thus, the challenge dose of procaine was carefully selected to provide a measurable response (duration of loss of righting ability) with a minimum of lethality. Potentiation of procaine-induced loss of righting ability (toxicity) was shown to increase as a function of the TOTP pretreatment dose. Statistical analysis showed a good correlation between esterase inhibition by TOTP and increased procaine toxicity.

Plasma has been suggested as the primary site of procaine hydrolysis in man [13], yet in other mammalian species procaine hydrolysis was greater in the liver than in plasma [14]. This is consistent with our results which indicate that mouse liver procaine esterase activity was approximately 100 times that of mouse plasma (Table 1). Although metabolism of procaine was measured under different pH conditions for these two tissues (pH 7.2 for liver, and pH 9.0 for plasma), 100 times may be a conservative estimate of the difference in tissue activities, since it has been suggested that pH 9 is optimum for esterase activity [15]. Thus, one might have expected an even greater difference had liver esterase activity been determined at pH 9. In this study, procaine metabolism in the mouse was used as a model for studying the possible

effects of organophosphate inhibition of liver esterase on the metabolism and toxicity of ester-containing drugs. There are many other drugs which are metabolized by liver esterases or closely related amidases. For example, isocarboxazid is hydrolyzed by liver amidases to a monoamine oxidase-inhibiting metabolite. EPN inhibited isocarboxazid hydrolysis and partially prevented isocarboxazid-induced inhibition of monoamine oxidase activity. [9].

At the time the present study was being completed, it was reported [10] that EPN pretreatment in rats increased procaine levels in both liver and kidney after procaine injection. On the other hand, after similar procaine injections, procaine concentrations were decreased in liver and kindey from rats which had been pretreated with enzyme-inducing doses of phenobarbital. That report lends support to our findings and together such studies provide evidence that organophosphate inhibition of carboxylesterase activity resulted in decreased ester drug hydrolysis, and a consequent increase in procaine toxicity *in vivo*.

The clinical implications of these findings are not yet clear. Additional studies involving other organophosphates and additional ester-containing drugs, are needed to determine which enzymes are most important for the metabolism of the various ester-containing drugs in man and other species likely to experience concomitant exposure to organophosphates and ester drugs. Such studies will determine the importance of monitoring carboxylesterase inhibition caused by organophosphates [16, 17] and may indicate that such monitoring should be included in studies to be used in the establishment of "safe levels" of organophosphates for daily consumption by man and other non-target species.

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