TOXICOLOGY AND APPLIED PHARMACOLOGY 73, 300–310 (1984)

Induction of Hepatic Microsomal Cytochrome *P*-450 and Inhibition of Brain, Liver, and Plasma Esterases by an Acute Dose of *S,S,S*-Tri-*n*-butyl Phosphorotrithioate (DEF) in the Adult Hen

DANIEL M. LAPADULA, CLARK D. CARRINGTON, AND MOHAMED B. ABOU-DONIA[1]

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

Received May 11, 1983; accepted November 28, 1983

Induction of Hepatic Microsomal Cytochrome *P*-450 and Inhibition of Brain, Liver, and Plasma Esterases by an Acute Dose of *S,S,S*-Tri-*n*-butyl Phosphorotrithioate (DEF) in the Adult Hen. LAPADULA, D. M., CARRINGTON, C. D., AND ABOU-DONIA, M. B. (1984). *Toxicol. Appl. Pharmacol.* **73**, 300–310. The differential effects of oral and dermal administration of single doses of 100 to 1000 mg/kg *S,S,S*-tri-*n*-butyl phosphorotrithioate (DEF) on nonspecific esterases and liver metabolism enzymes were investigated one day following administration. *O,O*-Diethyl *O*-(4-nitrophenyl) phosphorothioate (parathion) and tri-*o*-cresyl phosphate (TOCP) were used as negative and positive controls for organophosphorus-induced delayed neurotoxicity (OPIDN). Brain acetylcholinesterase was significantly inhibited with topical doses of 500 and 1,000 mg/kg of DEF and with orally and dermally applied parathion. Plasma cholinesterase and liver microsomal carboxylesterase activities were significantly reduced from control in all treatment groups. Neurotoxic esterase (NTE) was significantly decreased from control with topical dosing of 200, 500, and 1000 mg/kg DEF and with TOCP treatments. Oral doses of DEF increased cytochrome *P*-450 content by 70 to 200% while dermal application caused a 200 to 325% increase over control. *p*-Chloro-*N*-methylaniline demethylase was also increased by DEF treatments but to a lesser extent than that of aniline hydroxylase or cytochrome *P*-450 content. TOCP and parathion had no significant effect on liver microsomal oxidative enzymes. Liver microsomal proteins from hens treated with phenobarbital (PB), 3-methylcholanthrene (3MC), or DEF were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A striking increase in a 49K protein band in microsomes from PB and DEF (616 and 338%, respectively) treated hens was seen, while the 55K protein band showed an 861% increase in microsomes from 3MC-treated hens. In conclusion, dermally applied DEF was more effective in inhibiting esterases and inducing cytochrome *P*-450 than orally administered DEF; toxicity was directly related to the dose and route of administration.

Organophosphorus pesticides possess anticholinesterase properties, and some also produce delayed neurotoxicity (Cavanagh, 1973; Abou-Donia, 1981). Humans and some animal species (cats, dogs, cows, and chickens) are susceptible to organophosphorus-induced delayed neurotoxicity (OPIDN), while others (rodents and some primates) are not (Smith *et al.*, 1930; Abou-Donia, 1981). Clinical signs of OPIDN are characterized by a delay period of 6 to 14 days, after which ataxia ensues followed by paralysis. The neuronal lesions seen are degeneration of axons with subsequent Wallerian-type degeneration of myelin in the central and peripheral nervous systems.

S,S,S-Tri-*n*-butyl phosphorotrithioate (DEF), an aliphatic thiophosphate ester which is used as a cotton defoliant, produces both cholinergic and delayed neurotoxic effects in hens. A third type of toxicity is also seen in

[1] To whom requests for reprints should be addressed.

0041-008X/84 $3.00

DEF-treated hens. A late acute effect results after oral administration and is neither related to inhibition of acetylcholinesterase nor associated with histopathologic changes in nervous tissues. The neurotoxic effect is related to the dose and route of administration of DEF (Abou-Donia *et al.*, 1979a,b). For example, DEF is metabolized to *n*-butyl mercaptan (*n*BM) in the gastrointestinal tract (Abou-Donia *et al.*, 1979a), and the hematotoxic effect of *n*BM may contribute to the development of the late acute effect (Abdo *et al.*, 1983). By contrast, delayed neurotoxicity has been produced after dermal, intraperitoneal, or subcutaneous administration of DEF (Casida *et al.*, 1963; Baron and Johnson, 1964; Gaines, 1969; Abou-Donia *et al.*, 1979a,b).

Since DEF affects specific enzymes in the nervous system and plasma, and since its toxicologic manifestations seem to be influenced by metabolism enzymes, this study was carried out to investigate the effects of oral and dermal administration of DEF on these enzymes. The enzymes evaluated in this study were those demonstrated to be involved in the acute cholinergic toxicity (e.g., brain acetylcholinesterase and plasma butyrylcholinesterase), in delayed neurotoxicity (e.g., neurotoxic esterase), and in the metabolism of organophosphorus compounds (e.g., liver microsomal cytochrome *P*-450, aniline hydroxylase, *p*-chloro-*N*-methyl aniline demethylase, and carboxylesterase; Murphy *et al.*, 1975). Qualitative and quantitative analyses of microsomal proteins were carried out to determine which form of cytochrome *P*-450 was being induced.

METHODS

Chemicals

Technical grade *S,S,S*-tri-*n*-butyl phosphorotrithioate (DEF, 95%) was provided by Mobay Chemical Corp., Kansas City, Missouri. Tri-*o*-cresyl phosphate (TOCP, 99%) and *O,O*-diethyl *O*-4-nitrophenyl phosphorothioate (parathion, 99%) were obtained from Eastman Kodak Co., Rochester, New York, and Pflatz and Bauer, Inc., Stamford, Connecticut, respectively. The following enzyme substrates were purchased from the corresponding sources: aniline hydrochloride (99%), Fisher Chemical Co., Raleigh, North Carolina; *p*-chloro-*N*-methyl aniline (99%), Aldrich Chemical Co., Metuchen, New Jersey; reduced nicotine adenine denucleotide phosphate (NADPH, 97%), P-L Biochemicals, Inc., Milwaukee, Wisconsin; acetylthiocholine, butyrylthiocholine, and 1-naphthyl acetate, Sigma Chemical Co., St. Louis, Missouri.

Hens

Laying leghorn hens (*Gallus gallus* domesticus), 14 months old, weighing approximately 1.8 kg (1.45 to 2.15 kg), were used. The hens were specified to be pathogen free. Water and feed were provided *ad libitum* until termination. The birds were placed in stainless-steel cages, in humidity- (40 to 60%), and temperature- (21 to 23°C) controlled rooms with a 12-hr light cycle.

Treatment Protocols

Effect of organophosphorus compounds. Each of the following doses was orally or dermally administered to a group of five hens: 100, 200, 500, and 1000 mg/kg DEF; 500 mg/kg TOCP; or 5 mg/kg parathion. A group of five untreated hens was used as a control. Oral doses were given in gelatin capsules while topical administrations were applied with a micropipette over an area of 10 cm^2 on the back of the hen's unprotected neck. All hens were decapitated 24 hr after treatment.

Effect of microsomal enzyme inducers. Groups of three hens were treated with a daily intraperitoneal dose, for 3 consecutive days, of 80 mg/kg sodium phenobarbital (PB) in saline or 40 mg/kg 3-methylcholanthrene (3MC) in corn oil. A single dermal dose of 1000 mg/kg DEF was given to a group of three hens. Controls consisted of three untreated hens. All hens were killed by decapitation 24 hr after the last dose.

Enzymatic Analysis

Immediately after decapitation of the bird, brain, spinal cord, and liver were removed quickly. Spinal cords were removed by the deSousa and Horrocks (1979) method of injecting ice cold 0.9% saline into the vertebral canal.

Brain and plasma enzymes. Brain acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BuChE) were measured as described previously (Abou-Donia *et al.*, 1979a) and expressed as micromoles of acetylthiocholine (ATCH) or butyrylthiocholine (BUTCH) hydrolyzed per minute per milligram of protein, respectively. Neurotoxic esterase (NTE) was determined by the Johnson (1977) method and expressed as micromoles of phenyl valerate hydrolyzed per minute per milligram of protein.

Liver microsomal enzymes. Washed liver microsomes

were prepared from all hens according to the Schenkman and Cinti (1978) method, frozen with liquid nitrogen, and stored at −70°C. The resulting microsomal suspension contained 2 to 5 mg of protein per milliliter. Cytochrome *P*-450 content was assessed by the Omura and Sato (1964) method and expressed as micromoles of *P*-450 per milligram of protein.

The following microsomal enzymes were assayed in livers from hens treated with organophosphorus compounds and their control group: aniline hydroxylase, *p*-chloro-*N*-methylaniline (PCMA) demethylase, and carboxylesterase. Aniline hydroxylase activity was measured by the Mazel (1971) method with 1.5 μmol of NADPH replacing the NADPH generating system. Aniline hydroxylase was expressed as micromoles of *p*-aminophenol formed per milligram of protein per minute. Microsomal oxidative demethylation of PCMA (PCMA demethylase) was assayed according to the Abou-Donia and Dieckert (1971) method and expressed as micromoles of *p*-chloroaniline (PCA) formed per minute per milligram of protein. Carboxylesterase activity was determined by the Murphy *et al.* (1975) method with 1-naphthyl acetate as a substrate and a 1:10 dilution of microsomal proteins in the phosphate buffer (pH 7.4). This nonspecific esterase activity was expressed as nanomoles of 1-naphthol formed per minute per milligram of protein.

Protein. Proteins were determined in brain, plasma, and liver microsomes according to the Bradford (1976) method with bovine plasma gamma globulin (Bio-Rad Laboratories, Richmond, Calif.) as standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Microsomal proteins from livers of hens treated with microsomal enzyme inducers were analyzed by SDS–PAGE. Microsomal proteins were resolved on 10-well 0.1% SDS–polyacrylamide vertical slab gels (10 × 16 × 0.15 cm) (Bio-Rad Laboratories). Electrophoresis was performed by a modification of the Laemmli (1970) procedure with 4 and 7.5% acrylamide in the stacking and resolving gels, respectively. Liver microsomal samples from all treated and control hens were prepared with a final concentration of 100 μg protein/150-μl sample containing 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, and 0.125 M Tris–HCl followed by heating for 5 min in a 90°C water bath. Molecular weight markers consisted of ovalbumin (45K), bovine serum albumin (66.2K), phosphorylase B (92.5K), β-galactosidase (116.2K), and myosin (205K). Aliquots (150 μl) of the samples were subjected to electrophoresis under conditions of constant power (6 W per slab) until the tracking dye reached the bottom of the gel. Gels were fixed in 10/45/45 acetic acid/methanol/water, stained with 0.1% Coomassie blue in the same solvent mixture, and destained in fixing solution. Gels were soaked for 1 hr in 1/10/90 glycerol/acetic acid/water and dried on dialysis membrane. Microdensitometry was performed with an LKB Laser densitometer with a recording integrator (LKB Instruments, Inc., Bromma, Sweden).

Statistics

Significance of the difference between control and treated birds was assessed by Student's two-tailed *t* test. A *p* value of 0.05 or less was considered significant.

RESULTS

Clinical and Necropsy Observations

Hens administered dermal doses or large (500 and 1000 mg/kg) oral doses of *S,S,S*-tri-*n*-butyl phosphorotrithioate (DEF) or parathion exhibited signs of acute cholinergic toxicity (excess salivation and diarrhea) within 24 hr of dosing. Hens treated with 500 mg/kg tri-*o*-cresyl phosphate (TOCP) did not show acute signs of poisoning. No clinical signs characteristic of OPIDN or late acute effects were observed in any of the treated hens 1 day after treatment. No differences were detected between tissues of treated and control hens when compared for size, color, and shape at necropsy.

Brain and Spinal Cord Enzymatic Activities

Brain acetylcholinesterase (AChE). Hens given a single administration of DEF, either oral or topical, exhibited decreased AChE activity (Fig. 1). Dermal treatment of hens had the greatest effect, with doses of 500 and 1000 mg/kg resulting in significant inhibition on AChE. TOCP-treated animals exhibited a decrease in AChE activity. Parathion treatment, the positive control for the inhibition of AChE activity, effected approximately 20 to 25% inhibition, as compared with the control level.

Brain neurotoxic esterase (NTE). NTE activity was similar to AChE activity in DEF-treated animals (Fig. 2A), except with small oral doses of DEF. Topical doses of 100 and 200 mg/kg showed a definite decrease in NTE activity; the same amounts given orally did

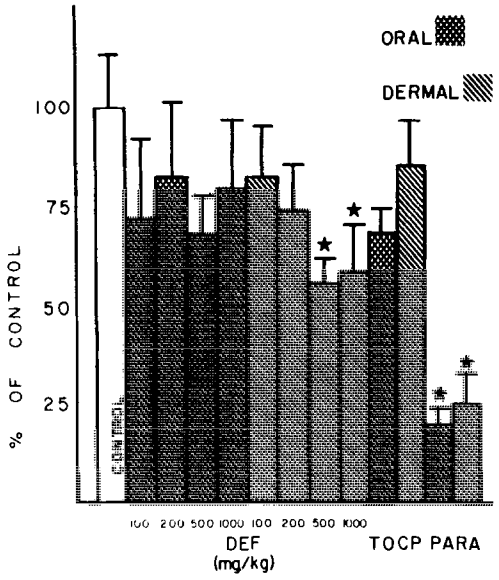

FIG. 1. The effect of DEF, TOCP, and parathion (PARA) on AChE. Control activity was 50.76 ± 6.79 (mean ± SE) μmol ATCH hydrolyzed/min/mg of protein. Values are means of values from at least four hens. (★) Significant difference from control. Vertical lines are SE.

not affect NTE activity. At all dose levels of DEF, animals treated dermally exhibited a greater decrease in NTE activity than orally treated animals, and at 200, 500, and 1000 mg/kg the decrease from control levels was statistically significant. TOCP significantly inhibited NTE activity; dermal administration had a greater apparent inhibitory effect than oral administration ($p < 0.05$). Parathion had no apparent effect on NTE activity.

Spinal cord NTE. In the spinal cord, NTE activity was significantly depressed with dermal doses of 500 and 1000 mg/kg DEF, but all other dose levels caused no significant difference from the control levels (Fig. 2B). TOCP also significantly inhibited spinal cord NTE, the oral administration being more effective than the dermal.

Plasma Butyrylcholinesterase (BuChE)

Plasma BuChE was significantly depressed from control levels by all treatments (Fig. 3). In general, dermal application of DEF inhibited BuChE more than did oral treatments. In all dermal DEF treatments, BuChE was inhibited to less than 20% of the control level.

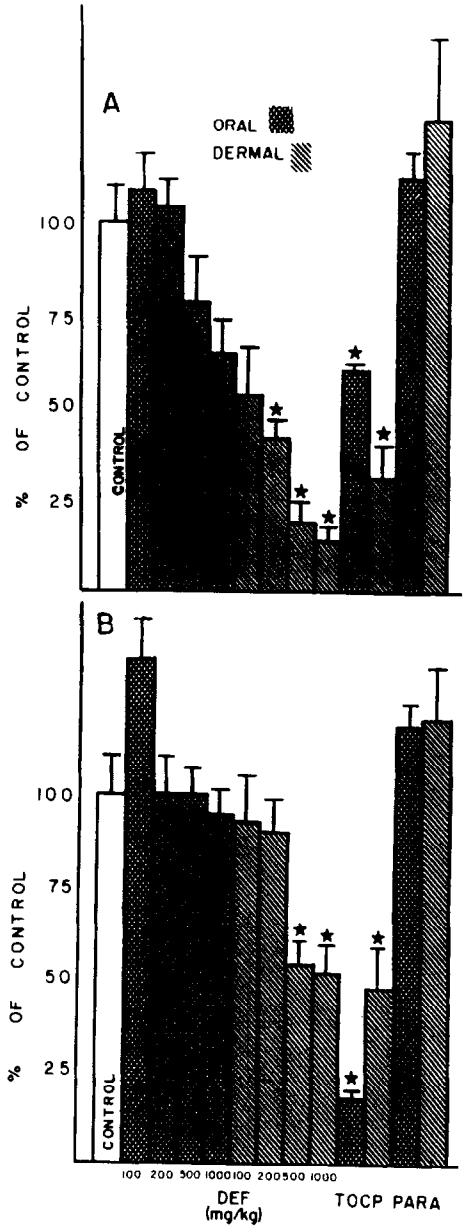

FIG. 2. Brain (A) and spinal cord (B) neurotoxic esterase (NTE) activity. Brain and spinal cord control NTE activity was 23.77 ± 2.35 and 6.65 ± 0.65 nmol of phenylvalerate hydrolyzed/min/mg of protein, respectively. (★) Significant difference from control. Values are means of values from at least four hens. PARA is parathion. Vertical lines are SE.

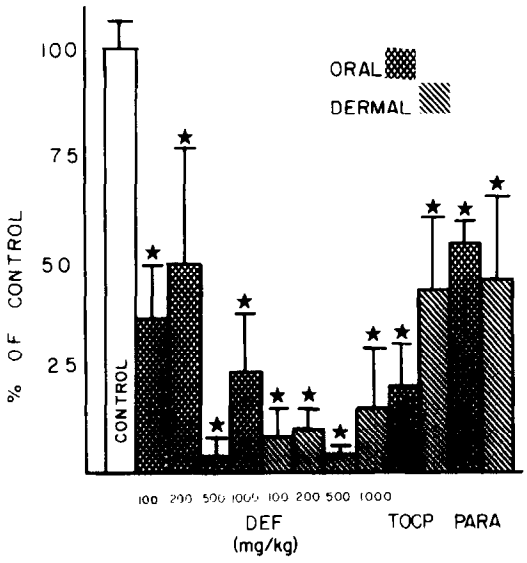

FIG. 3. Plasma BuChE activity in DEF, TOCP, and parathion treated animals. Control activity is 1.47 ± 0.10 μmol BUTCH-hydrolyzed/min/mg of protein. (★) Significant difference from control. Values are means of values from at least three hens. PARA is parathion. Vertical lines are SE.

Orally administered TOCP inhibited BuChE to 20% of the control while dermal administration inhibited BuChE to 45% of control; however, these values were not significantly different from each other. Parathion inhibited BuChE the least, 45 and 54% of control, topically and orally, respectively.

Microsomal Enzymes

Cytochrome P-450. Liver microsomes from untreated hens contained only small amounts of cytochrome *P*-450, i.e., 0.123 ± 0.039 nmol/mg of proteins. Cytochrome *P*-450 content in microsomes from DEF-treated animals was induced in a dose- and route-dependent manner (Fig. 4). Specifically, microsomes from animals given an oral dose of 1000 mg/kg DEF exhibited the same cytochrome *P*-450 content as those from animals treated with a dermal 100-mg/kg dose of DEF, the content after treatment producing a 200% increase over the control. When an oral dose of 100 mg/kg of DEF was given, the cytochrome *P*-450 content rose to only a 73% increase over control. Single dermal and oral doses of TOCP and parathion exhibited no effect on cytochrome *P*-450 content.

p-Chloro-N-methylaniline (PMCA) demethylase. PCMA oxidative demethylation did not exhibit the marked increase demonstrated with aniline hydroxylase (Fig. 5A). The only significant increase over the control was with dermal DEF treatments of 200 and 500 mg/kg, although all treatments appeared to show an increase over control activity. Neither TOCP nor parathion affected either aniline hydroxylase activity or oxidative demethylation of PCMA.

Aniline hydroxylase. Increases in microsomal aniline hydroxylase activity paralleled those seen with cytochrome *P*-450 concentrations (Fig. 5B). Although the dose dependence was not as prominently seen in the aniline hydroxylase activity, the route of ad-

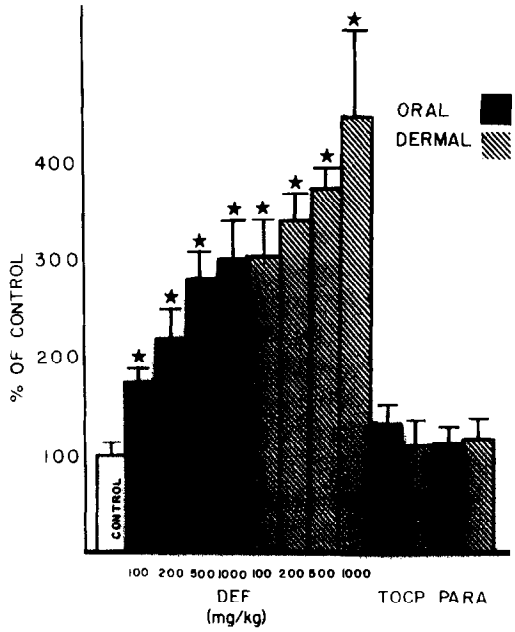

FIG. 4. Cytochrome *P*-450 content in liver microsomes. Control cytochrome *P*-450 content is 0.123 ± 0.039 nmol/mg of protein. (★) Significant difference from control. Values are means of values from at least four hens. PARA is parathion. Vertical lines are SE.

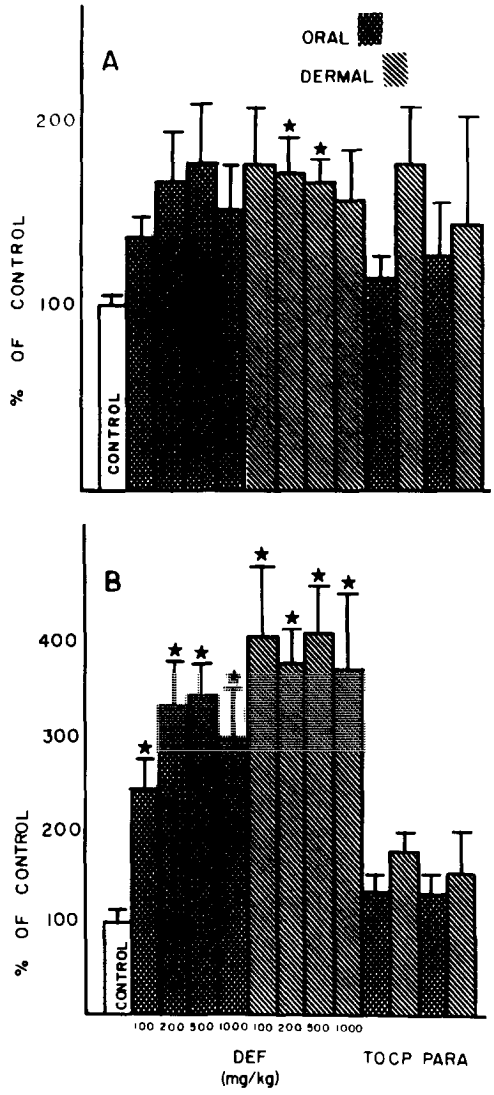

FIG. 5. PCMA demethylase (A) and aniline hydroxylase (B) activity of liver microsomes. Control values are 0.342 ± 0.016 nmol of *p*-chloroaniline formed/min/mg of protein and 0.357 ± 0.020 nmol of *p*-aminophenol formed/min/mg of protein, respectively. (★) Significant difference from control. Values are means of values from at least four hens. PARA is parathion. Vertical lines are SE.

ministration still played a role. Dermal application of DEF increased aniline hydroxylase activity 200 to 300% over the control while oral administration increased the activity 100 to 200%.

Carboxylesterase. Carboxylesterase, a nonspecific esterase with no known critical physiological function, decreased with DEF treatment (Fig. 6). The decrease was dependent upon both the dose of DEF and its route of administration. Dermal treatments were more effective than oral treatments of DEF, with the greatest decrease occurring with a dermal treatment of 1000 mg/kg (82% inhibition). Both TOCP and parathion administrations significantly inhibited microsomal carboxylesterase activity.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Coomassie blue staining revealed distinct differences between microsomal protein profiles from 3-methylcholanthrene- (3MC), phenobarbital- (PB), and DEF-treated animals and from untreated hens (Fig. 7). Microdensitometry demonstrated that a significant increase in protein of 49K, 52K, and 53.5K bands occurred in microsomes from animals treated with PB (Fig. 7, Table 1). Microsomes from 3MC-treated animals showed a significant increase in 52K and 55K bands. A com-

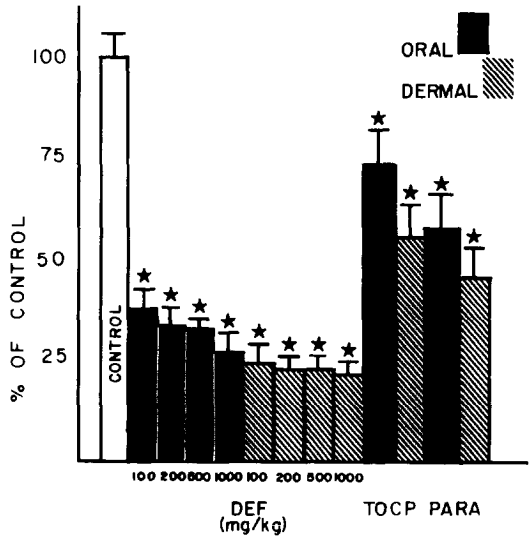

FIG. 6. Carboxylesterase of liver microsomes. Control activity is 1651 ± 92 nmol of α-napthol formed/min/mg of protein. (★) Significant difference from control. All values are for at least four hens. PARA is parathion. Vertical lines are SE.

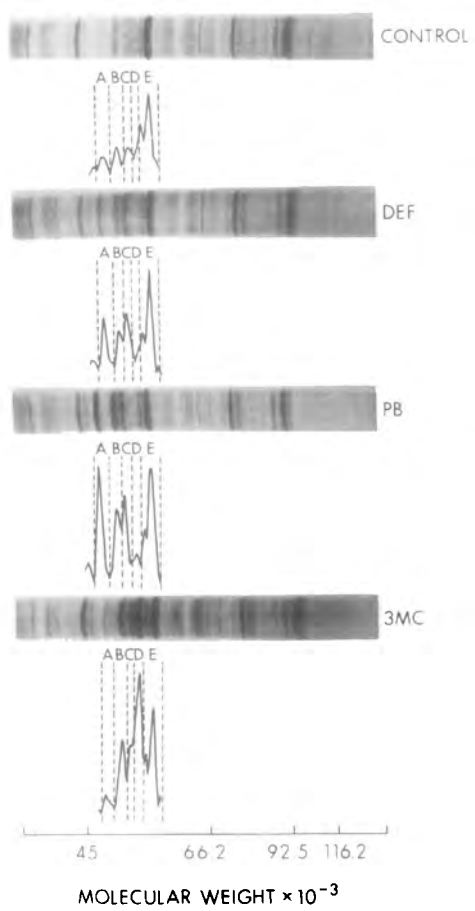

FIG. 7. Gels and microdensitometric scans of microsomes from animals treated with DEF, PB, and 3MC. Band A, M_r = 49K is the putative cytochrome *P*-450. Band D, M_r = 55K is the putative cytochrome *P*-448. In DEF-treated animals, note the increased band A indicating an increase in cytochrome *P*-450. Bands B, M_r = 52K, and C, M_r = 53.5K, were increased with all treatments. Band E, M_r = 58.5K was present in all gels and was used as an internal marker. The cytochrome *P*-450 content in these gels were (nmol/mg of protein): Control, 0.121; DEF, 0.320; PB, 0.488; 3MC, 0.610.

parison of microsomes from DEF-treated animals revealed a pattern similar to that of PB-treated animals, a finding indicative of an induction of cytochrome *P*-450.

Scans of these microsomes for cytochrome *P*-450 content revealed a λ_{max} of the Soret peak of the reduced cytochrome *P*-450 CO complex in PB- and DEF-treated animals to be 450 nm while it was 448 nm in 3MC-treated animals and 451 nm in control animals.

DISCUSSION

S,S,S-Tri-*n*-butyl phosphorotrithioate (DEF) is a cotton defoliant known to produce organophosphorus-induced delayed neurotoxicity (OPIDN) in chickens after dermal administration (Abou-Donia *et al.*, 1979a,b). Two other toxicological effects of DEF are known: acute cholinergic toxicity and the late acute effect. This study investigated enzymes involved in the metabolism and toxicity of DEF.

The acute toxicity of organophosphorus compounds, in general, is due to the inhibition of acetylcholinesterase (AChE). In addition carboxylesterase and/or butyrylcholinesterase (BuChE) may also be involved in the toxicologic effects, although neither has a known critical physiological function. The present study demonstrates that DEF is a potent inhibitor of both carboxylesterase and BuChE; this result correlates with the findings of other investigators who used different species or dosing regimens (Murphy and DuBois, 1959; Murphy *et al.*, 1975; Abou-Donia *et al.*, 1979a,b).

TABLE 1

QUANTITATION OF PROTEIN BANDS FROM SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS TREATMENTS EXPRESSED AS A PERCENTAGE OF CONTROL

Protein band	Treatment		
	3MC[a]	PB[b]	DEF
49K (A)	112 ± 14	616 ± 30*	338 ± 37*
52K (B)	179 ± 22*	255 ± 13*	152 ± 18
53.5K (C)	160 ± 18	272 ± 27*	224 ± 38*
55K (D)	816 ± 91*	73 ± 15	78 ± 25

[a] 3MC = 3-methylcholanthrene.

[b] PB = phenobarbital.

* Significantly different from control values ($p < 0.05$, $\bar{x} \pm$ SE, $n = 3$.

This study showed DEF to be an effective inhibitor of brain AChE at topical doses of 500 and 1000 mg/kg, which accounts for the acute toxicity observed after single doses of DEF (Abou-Donia *et al.*, 1979a,b). As demonstrated by Murphy and Dubois (1959) in the rat, DEF is also a more potent inhibitor of BuChE than AChE in the chicken. In general, organophosphorus esters that cause OPIDN inhibit BuChE more effectively than AChE (Barnes and Denz, 1953); however, BuChE is not directly involved in the development of delayed neurotoxicity. Topical administration has a prolonged effect on BuChE activity as compared with oral ingestion (Abou-Donia *et al.*, 1979a). The inhibition of carboxylesterase by DEF is consistent with other studies (Murphy *et al.*, 1975). Although the time course of the inhibition and reactivation of both carboxylesterase and BuChE following DEF administration is unknown, the binding of organophosphorus esters to these esterases may reduce their circulating pool (Abou-Donia, 1981).

This laboratory has previously demonstrated that topical doses of DEF are more effective in producing OPIDN than oral doses. In this study, dermally administered DEF was an effective inhibitor of neurotoxic esterase (NTE) at higher dose levels, while NTE was not inhibited by orally administered doses. This finding correlates with the clinical and histopathological studies of chickens given single doses of DEF, both topical and oral (Abou-Donia *et al.*, 1979a,b). Although the inhibition of NTE is correlated with the production of OPIDN, the mechanism is unknown. Previous studies showed a relationship between inhibition and "aging" of NTE by organophosphorus compounds and the ability of these chemicals to produce OPIDN in hens (Johnson, 1982). NTE has not been isolated, however, and its physiologic function is not known. Furthermore, NTE is present, inhibited, and aged in young chicks and in rats without subsequent development of OPIDN (Johnson, 1981).

The present results demonstrate that inhibition of the three nonspecific esterases is associated with the DEF dose and the dermal route of entry that produces OPIDN. Thus, parathion and orally administered DEF did not cause OPIDN and did not inhibit these esterases to the same extent as did TOCP and dermally applied DEF, both of which are capable of producing OPIDN.

It is interesting to speculate on the role of nonspecific esterases (e.g., BuChE, NTE, and carboxylesterase) in the pathogenesis of OPIDN. None of these enzymes has a known physiologic function; yet, delayed neurotoxic organophosphorus compounds inhibit these enzymes (especially BuChE and NTE) to a much greater extent that other organophosphorus compounds do. One can speculate that a high affinity for these esterases initially protects the exposed hens from the acute effect of these chemicals by reducing the circulating pool. These hens would survive long enough for the organophosphorus chemicals to be freed, either by a reversible process or in the course of the turnover of these enzymes (Clement, 1982), and as time passes, to accumulate at the neurotoxicity target until they reach the minimum amount or threshold that causes OPIDN (Abou-Donia, 1981).

Another interesting finding of this study is the marked induction of cytochrome *P*-450 content. This result following DEF administration was confirmed with sodium dedocyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The induction of cytochrome *P*-450 was both dose and route dependent. Aniline hydroxylase and *p*-chloro-*N*-methylaniline (PCMA) demethylase activity, which are both cytochrome *P*-450 mediated reactions, also increased with oral and dermal administrations of DEF. Neither TOCP nor parathion produced a significant induction of cytochrome *P*-450 content, or aniline hydroxylase or PCMA demethylase activity. These results are consistent with a previous study of the delayed neurotoxic insecticide *O*-ethyl *O*-4-nitrophenyl phenylphosphono-

thioate (EPN), in which a single oral dose of 100 mg/kg administered to hens showed a 100% increase in cytochrome *P*-450 content and EPN metabolism. However, three consecutive doses of 15 mg/kg daily did not induce either cytochrome *P*-450 content or EPN metabolism (Lasker *et al.*, 1982). It is not known whether this previous study with EPN yielded the same form of cytochrome *P*-450 as was found in the present study of DEF.

SDS–PAGE revealed a staining pattern for cytochrome *P*-450 that was similar to that demonstrated in rats (Welton and Aust, 1974; Moore *et al.*, 1978). Welton and Aust (1974) found an increase in 44K and 47K bands in phenobarbital- (PB) treated animals and 53K and 50K bands in 3-methylcholanthrene- (3MC) treated animals. Moore *et al.* (1978) confirmed an increase in a 45K band in PB-treated animals and a 53K band in 3MC-treated animals. The results in the study reported here are similar to these findings: PB increased the lower M_r band (49K) the most, while 3MC increased the higher M_r band (55K). Since DEF increased the same bands as did PB, the induction of a PB-type cytochrome *P*-450 is indicated. The present results also confirm previous findings that chicken liver microsomal cytochrome *P*-450 content is approximately one-fourth that of the rat (Lasker *et al.*, 1982; Ehrich and Larsen, 1983). This study, however, does not show which form of cytochrome *P*-450 was induced. Guengerich (1978, 1979) has demonstrated that at least six forms of cytochrome *P*-450 can be induced in the rat with phenobarbital, with at least three more fractions present in 3-methylcholanthrene-treated or untreated rats. Many of these forms of cytochrome *P*-450 had the same apparent molecular weight on SDS–PAGE. It is not known how many possible forms of cytochrome *P*-450 are present in the chicken. Further work on the characterization of cytochrome *P*-450 in the chicken is necessary to determine which forms were induced with either phenobarbital or DEF.

The induction of cytochrome *P*-450 may in part explain the observation of Murphy *et al.* (1975) that there was less potentiation of Guthion (*O,O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl]phosphorodithioate) toxicity by orally administered DEF than with DEF administered by ip injection. One can speculate that the desulfuration of Guthion to Guthoxon (*O,O*-dimethyl *S*[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl]phosphorothioate) (DuBois *et al.*, 1957), which is probably mediated by cytochrome *P*-450, is enhanced in DEF-treated animals in a route-dependent manner. Since the enhanced toxicity of Guthion was dependent upon the route of administration of DEF, a possible mechanism of its potentiation by DEF would be that the cytochrome *P*-450 was induced to a greater extent when given intraperitoneally than with oral administration.

The route of entry also effects the toxicity of DEF. The present study demonstrates a differential toxicity of oral versus dermal treatment of animals with DEF. A number of factors may play a role, among them differences in absorption, metabolism, and disposition of DEF and its metabolites, such as those found with leptophos (Abou-Donia, 1976, 1979, 1980, 1983). Nevertheless, it is apparent that dermal administration of DEF is more effective in the inhibition of esterases and the induction of hepatic cytochrome *P*-450 and oxidases than is oral administration. Studies are currently underway in this laboratory to investigate the metabolism and disposition of DEF (Nomeir and Abou-Donia, 1983). Preliminary results indicate that an active metabolite of DEF, DEF–sulfoxide, is formed by chicken hepatic microsomal cytochrome *P*-450. This metabolism might account for the induction of cytochrome *P*-450 and the marked increase of *in vivo* inhibition of AChE with DEF. Similar metabolic activation was demonstrated with TOCP (Eto *et al.*, 1962). The enhanced effectiveness of DEF with dermal exposure might be attributed to the persistence of DEF or its active metabolite in the

hen. Previous studies in this laboratory have demonstrated that after a 400-mg/kg dose of DEF, plasma concentration of *n*-butyl mercaptan (*n*BM), a hydrolysis product of DEF, was 52 μg/ml when orally administered or 7.2 μg/ml when topically applied (Abou-Donia *et al.*, 1979b). These results indicated a greater hydrolysis of orally applied DEF. In agreement with these results is the finding that following oral administration of 400 mg/kg DEF in hens, plasma BuChE activity returned to the control value in 12 days, while plasma BuChE activity returned only to 50% of the control value 30 days after the topical administration of the same dose. These earlier findings are supported by the recent pharmacokinetic studies of a single oral or dermal dose of 50 mg/kg [^{14}C]DEF in hens, which showed that the half-life of ^{14}C in plasma was 2.7 or 3.8 days, respectively (Abou-Donia *et al.*, 1983). All of these studies indicate that an oral dose of DEF is metabolized and excreted much more rapidly than the topically administered compound. In general, the parent compound or an active metabolite was present in the hen for a longer period of time after topical administration, which may account for the difference in oral versus dermal toxicity.

Our findings that dermal administration of DEF is a more effective inhibitor of esterases and is more likely to produce delayed neurotoxicity (Abou-Donia *et al.*, 1979b) indicate that topical administration, as well as the oral dosing required by the Environmental Protection Agency (Anonymous, 1978), should be done in any testing of DEF and of organophosphorus compounds in general.

ACKNOWLEDGMENTS

The authors thank Mrs. Erna S. Daniel for her secretarial work in the preparation of this manuscript and Dr. Elmer J. Rauckman for assistance in cytochrome *P*-450 content determinations. The supply of technical DEF from Mobay Chemical Corp. Kansas City, Missouri, is acknowledged. This study was supported in part by NIEHS Grant ES02717, NIOSH Grant OH00823, and NIEHS Training Grant T32 ES07031.

REFERENCES

ABDO, K. M., TIMMONS, P. R., GRAHAM, D. G., AND ABOU-DONIA, M. B. (1983). Heinz body production and hematological changes in the hen after administration of a single oral dose of *n*-butyl mercaptan and *n*-butyl disulfide. *Fundam. Appl. Toxicol.* **3,** 69–74.

ABOU-DONIA, M. B. (1976). Pharmacokinetics of a neurotoxic oral dose of leptophos in hens. *Arch. Toxicol.* **36,** 103–110.

ABOU-DONIA, M. B. (1979). Pharmacokinetics and metabolism of a topically applied dose of *O*-4-bromo-2,5-dichlorophenyl *O*-methyl phenylphosphonothioate in hens. *Toxicol. Appl. Pharmacol.* **51,** 311–328.

ABOU-DONIA, M. B. (1980). Metabolism and pharmacokinetics of a single oral dose of *O*-4-bromo-2,5-dichlorophenyl *O*-methyl phenylphosphonothioate (leptophos) in hens. *Toxicol. Appl. Pharmacol.* **55,** 131–145.

ABOU-DONIA, M. B. (1981). Organophosphorus ester induced delayed neurotoxicity. *Annu. Rev. Pharmcol. Toxicol.* **21,** 511–548.

ABOU-DONIA, M. B. (1983). Toxicokinetics and metabolism of delayed neurotoxic organophosphorus esters. *Neurotoxicology* **4,** 113–130.

ABOU-DONIA, M. B., AND DIECKERT, J. W. (1971). Gossypol: Subcellular localization and stimulation of rat liver microsomal oxidases. *Toxicol. Appl. Pharmacol.* **18,** 507–516.

ABOU-DONIA, M. B., GRAHAM, D. G., ABDO, K. M., AND KOMEIL, A. A. (1979a). Delayed neurotoxic, late acute and cholinergic effects of *S,S,S*-tributyl phosphorotrithioate (DEF): Subchronic (90 days) administration in hens. *Toxicology* **14,** 229–243.

ABOU-DONIA, M. B., GRAHAM, D. G., TIMMONS, P. R., AND REICHERT, B. L. (1979b). Delayed neurotoxic and late acute effects of *S,S,S*-tributyl phosphorotrithioate on the hen: Effect of route of administration. *Neurotoxicology* **1,** 425–447.

ABOU-DONIA, M. B., LAPADULA, D. M., CARRINGTON, C. D., AND NOMEIR, A. A. (1983). The significance of inhibition of nonspecific esterases in the development of organophosphorus-induced delayed neurotoxicity. *Proceedings of the Second International Meeting on Cholinesterases* (in press).

Anonymous (1978). Environmental Protection Agency. Pesticides Programs. Proposed guidelines for registering pesticides in the U.S. hazard evaluation: Health and Domestic animal. Acute delayed neurotoxicity study. *Fed. Reg.* **43,** 37362–37363.

BARNES, J. M., AND DENZ, F. A. (1953). Experimental demyelination with organophosphorus compounds. *J. Pathol. Bacteriol.* **65,** 597–605.

BARON, R. L., AND JOHNSON, H. (1964). Neurological disruption produced in hens by two organophosphate esters. *Brit. J Pharmacol.* **23,** 295–304.

BRADFORD, M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

CASIDA, J. E., BARON, R. L., ETO, M., AND ENGEL, J. L. (1963). Potentiation and neurotoxicity induced by certain organophosphates. *Biochem. Pharmacol.* **12,** 73–83.

CAVANAGH, J. B. (1973). Peripheral neuropathy caused by chemical agents. *CRC Crit. Rev. Toxicol.* **2,** 365–417.

CLEMENT, J. G. (1982). Plasma aliesterase—A possible depot for soman (pinacolyl methylphosphonofluoridate) in the mouse. *Biochem. Pharmacol.* **31,** 4085–4088.

DESOUSA, B. N., AND HORROCKS, L. A. (1979). Development of rat spinal cord. I. Weight and length, with a method for rapid removal. *Dev. Neurosci.* **2,** 115–121.

DUBOIS, K. P., THURSH, D. R., AND MURPHY, S. D. (1957). Studies on the toxicity and pharmacologic actions of the dimethoxy ester of benxotriazine dithiophosphoric acid (DBD, Guthion). *J. Pharmacol. Exp. Ther.* **119,** 208–218.

EHRICH, M., AND LARSEN, C. (1983). Drug metabolism in adult white leghorn hens—Response to enzyme inducers. *Comp. Biochem. Physiol.* **64C,** 383–386.

ETO, M., CASIDA, J. E., AND ETO, T. (1962). Hydroxylation and cyclization reactions involved in the metabolism of tri-*o*-cresyl phosphate. *Biochem. Pharmacol.* **11,** 337–352.

GAINES, J. B. (1969). Acute toxicity of pesticides. *Toxicol. Appl. Pharmacol.* **14,** 515–534.

GUENGERICH, F. P. (1978). Separation and purification of multiple forms of microsomal cytochrome P-450. *J. Biol. Chem.* **253,** 7931–7939.

GUENGERICH, F. P. (1979). Isolation and purification of cytochrome P-450, and the existence of multiple forms. *Pharmac. Ther.* **6,** 99–121.

JOHNSON, M. K. (1977). Improved assay for neurotoxic esterase of screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.* **37,** 113–115.

JOHNSON, M. K. (1981). Initiation of organophosphate neurotoxicity. *Toxicol. Appl. Pharmacol.* **61,** 480–481.

JOHNSON, M. K. (1982). The target for initiation of delayed neurotoxicity by organophosphorus ester: Biochemical studies and toxicological applications. *Rev. Biochem. Toxicol.* **4,** 141–202.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bactriophage T4. *Nature (London)* **277,** 680–685.

LASKER, J. M., GRAHAM, D. G., AND ABOU-DONIA, M. B. (1982). Differential metabolism of *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate by rat and chicken hepatic microsomes. *Biochem. Pharmacol.* **31,** 1961–1967.

MAZEL, P. (1971). Experiments illustrating drug metabolism *in vitro*. In *Fundamentals of Drug Metabolism and Drug Disposition.* (D. N. LaDu, H. G. Mandel, and E. L. Way, eds.), pp. 546–582. Williams & Wilkins, Baltimore, Md.

MOORE, R. W., WELTON, A. F., AND AUST, S. D. (1978). Detection of hemoproteins in SDS-Polyacrylamide Gels. In *Methods of Enzymology* (S. Fleischer and L. Packer, eds.), Vol. 52, pp. 324–331. Academic Press, New York.

MURPHY, S. D., AND DUBOIS, K. P. (1959). Toxicity and Anticholinesterase Activity of tributyl phosphorotrithioate (DEF). *Arch. Ind. Health* **20,** 161–166.

MURPHY, S. D., CHEEVER, K. L., CHOW, A. Y. K., AND BREWSTER, M. (1975). Organophosphate insecticides potentiation by carboxylesterase inhibitors. In *Proceedings, European Society for Toxicology* Vol. XVII Excerpta Medica Int. Conf. Ser. No. 376. pp. 292–300.

NOMEIR, A. A., AND ABOU-DONIA, M. B. (1983). Metabolism and disposition of a single oral dose of *S,S,S*-tri-*n*-butyl phosphorotrithioate (DEF) in hens. *Toxicol. Lett.* **18**(Suppl. I), 3.

OMURA, T., AND SATO, R. (1964). The carbon-monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239,** 2370–2378.

SCHENKMAN, J. B., AND CINTI, D. L. (1978). Preparation of microsomes with calcium. In *Methods of Enzymology* (S. Fleischer and L. Packer, eds.), Vol. 52, pp. 83–89. Academic Press, New York.

SMITH, M. I., ELVOVE, E., AND FRAZIER, W. H. (1930). The pharmacological action of certain phenol esters, with special reference to the etiology of so-called ginger paralysis. *Public Health Rep.* **45,** 2509–2524.

WELTON, A. R., AND AUST, S. D. (1974). Multiplicity of cytochrome P-450 hemoproteins in rat liver microsomes. *Biochem. Biophys. Res. Commun.* **56,** 898–806.