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COMPARISON OF NEUROTOXIC EFFECTS AND POTENTIAL RISKS FROM ORAL ADMINISTRATION OR INGESTION OF TRICRESYL PHOSPHATE AND JET ENGINE OIL CONTAINING TRICRESYL PHOSPHATE

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Neurotoxicity of tricresyl phosphates (TCPs) and jet engine oil (JEO) containing TCPs were evaluated in studies conducted in both rat and hen. Results for currently produced samples ("conventional" and "low-toxicity") were compared with published findings on older samples to identify compositional changes and relate those changes to neurotoxic potential. Finally, a human risk assessment for exposure by oral ingestion of currently produced TCPs in JEO at 3% (JEO + 3%) was conducted. TCPs and certain other triaryl phosphates administered as single doses inhibited brain neuropathy target esterase (B-NTE; neurotoxic esterase) in the rat and the hen (hen 3.25 times as sensitive), and both species were deemed acceptable for initial screening purposes. Neither rat nor hen was sensitive enough to detect statistically significant inhibition of B-NTE after single doses of JEO + 3% "conventional" TCP. Subacute administration of 2 g/kg/d of JEO + 3% "conventional" TCP to the hen produced B-NTE inhibition (32%), which did not result in organophosphorus-induced delayed neurotoxicity (OPIDN). Subchronic administration of JEO + 3% TCP but not JEO + 1% TCP at 2 g/kg/d produced OPIDN. Thus, the threshold for OPIDN was between 20 and 60 mg "conventional" TCP/kg/d in JEO for 10 wk. The current "conventional" TCPs used in JEO and new "low-toxicity" TCPs now used in some JEO are synthesized from phenolic mixtures having reduced levels of ortho-cresol and ortho-xylenols resulting in TCPs of very high content of meta- and para-substituted phenyl moieties; this change in composition results in lower toxicity. The "conventional" TCPs still retain enough inhibitory activity to produce OPIDN, largely because of the

> *presence of* ortho-*xylyl moieties; the "low-toxicity" TCPs are largely devoid of* ortho *substituents and have extremely low potential to cause OPIDN. The TCPs produced in the 1940s and 1950s were more than 400 times as toxic as the "low-toxicity" TCPs produced today. Analysis of the doses required to produce OPIDN in a subchronic hen study suggests that the minimum toxic dose of "conventional" TCP for producing OPIDN in a 70-kg person would be 280 mg/d, and for JEO containing 3% TCP, 9.4 g/d. Food products could be inadvertently contaminated with neat "conventional" TCP but it is unlikely that food such as cooking oil would be contaminated with enough JEO + 3% TCP to cause toxicity. Further, at the dosage required for neurotoxicity, it would be virtually impossible for a person to receive enough JEO + 3% TCP in the normal workplace (or in an aircraft) to cause such toxicity. There is no record of a JEO formulated with the modern "conventional" TCP causing human neurotoxicity.*

Tricresyl phosphate (TCP) is used in the formulation of lubricants as an antiwear additive to enhance load-carrying capacity (LCC) and tolerance to increasing speed of rotating or sliding motion. Although many lubricants contain TCP, as well as other triaryl phosphates (TAP) at concentrations considerably below 1%, jet turbine engine oils most commonly contain concentrations of approximately 3%. Typical commercial-grade TCP, available since the 1950s, is a complex mixture of structurally related compounds, some of which are potent neurotoxicants. A characteristic neuropathy, often referred to as organophosphate-induced delayed neurotoxicity (OPIDN), occurs in humans and several animal species, most notably cat and hen, after exposure to these agents. This potentially severe toxicological syndrome is characterized by a 7- to 28-d delay period prior to the onset of clinical signs, which include ataxia, flaccid paresis, and paralysis. These effects result from distal axonal degeneration of both motor and sensory axons (Spencer & Shaumburg, 1978). Compounds in TCP are also weak inhibitors of cholinesterase enzymes in blood and nervous system, but this inhibitory property is not associated with observed toxic effects. There have been many instances of human OPIDN caused by TCP, and perhaps more than 60,000 people have been poisoned worldwide (World Health Organization, 1990). Almost all of the cases have resulted from contamination of foods or the inadvertent use of TCP itself as a cooking oil. One major incident took place in Morocco in 1959 and involved about 10,000 cases. In this instance, ingestion of cooking oil adulterated with unused jet turbine engine oil containing approximately 3% TCP was responsible for the outbreak (Henschler & Neumann, 1968).

The neurotoxic components in TCP are produced from *ortho*-alkyl-substituted phenol or xylenol present in the reaction mixture used for the synthesis. *Ortho*-methyl (cresyl) or *ortho*-ethyl phenols lead to highly toxic components, whereas *ortho*-substituted xylenols lead to less toxic components (Henschler & Bayer, 1958). TCP synthesized from only *meta*- and *para*-cresols does not cause OPIDN, although it still retains anticholinesterase activity. The relationship between the chemical structure of

many pure triaryl phosphates and potency in causing OPIDN has been extensively studied, and there is a rather comprehensive knowledge of the relative neurotoxic activities of these compounds (Henschler, 1958, 1959; Neumann & Henschler, 1957; Henschler & Bayer, 1958; Johannsen et al., 1977; Metcalf, 1982; Bondy et al., 1960; Johnson, 1975).

The antiwear properties of TCP, and certain other triaryl phosphates, are unique to this class of compounds; therefore, complete replacement by other less toxic additives is not possible for many applications. Jet turbine engine oils must meet stringent performance requirements, and up to 7 yr of engine testing may be required to obtain approval for all engines and operating conditions. The molecular diversity of commercial TCP and impurities present have been thought to contribute to the antiwear properties (Davey, 1950; Klaus & Bieber, 1965). Pure tri-*para*-cresyl phosphate was also reported to lubricate less well than commercial TCP (Godfrey, 1965). These factors have caused lubricant formulators and manufacturers to use caution in replacing, for toxicology reasons, the TCP additives that perform well in critical applications.

However, since the 1950s manufacturers have taken steps to lower the levels of *ortho*-cresyl and *ortho*-ethylphenyl isomers in TCP to reduce the potential for neurotoxicity (McCormick et al., 1993). Nevertheless, despite the reductions in *ortho*-isomer content, scientists at the Mobil Environmental and Health Sciences Laboratory found in 1988 that a jet engine oil formulated with TCP markedly inhibited serum pseudocholinesterase and slightly inhibited erythrocyte and brain acetylcholinesterase activities in a rat 90-d dermal toxicity study. A subsequent single-dose oral study in male Long-Evans rats with neat TCP at 2 g/kg showed a 74% inhibition of brain neuropathy target esterase (NTE), suggesting the potential to cause OPIDN.* A follow-up 10-wk subchronic hen study was then conducted with representative formulated jet engine oils containing 0.5–3% of this TCP sample administered orally by gavage. In this study, 2 g/kg/d of the oil containing 3% TCP produced OPIDN (Freudenthal et al., 1993). These findings, and the large number of human poisonings that have occurred after TCP ingestion, suggest that in the past there has been a lack of understanding regarding human health risks from commercial TCPs.

Since 1988, an extensive screening and testing program was conducted by Mobil to identify and replace TAP additives in various lubricants, including jet turbine engine oils, which have the potential to cause OPIDN. Manufacturers of TCP have also continued their efforts to develop products of lower toxicity. In this article, the toxicological effects of several TCPs and other TAPs are presented and are related to chemical composition. The neurotoxic hazard and risk are estimated for TCP-containing lubricants produced over the past 50 yr.

*TSCA Section 8(e) notification, 27 June 1988, U.S. EPA Document Control No. 8EHQ-0788-0744S.

METHODS

Standards and Reagents

Tri-*ortho*-cresyl phosphate (TOCP), tri-*meta*-cresyl phosphate (TMCP), and tri-*para*-cresyl phosphate (TPCP) used as reference materials in the gas chromatography/mass spectroscopy (GC/MS) analysis of commerical TCPs and as positive and negative controls for enzyme inhibition studies were the highest purity available from Eastman Kodak. Mixed cresyl/xylyl and cresyl/ethylphenyl phosphate esters were synthesized in this laboratory. Butyrylcholine and acetylthiocholine substrates were purchased from Sigma Chemical Co. Phenyl valerate, paraoxon, and mipafox used in the NTE assays were obtained as a kit from Chem Science Laboratories, Lenexa, KS.

Aryl phosphate samples were obtained from eight commercial suppliers (Table 1). All of the samples were synthesized from complex mixtures of methyl phenols (cresols), dimethyl phenols (xylenols), phenol, other alkylated phenols, and small amounts of unidentified impurities. In this article, all samples tested, with the exception of the TCP type, are referred to as triaryl phosphates (TAPs).

Animals

Male Long-Evans rats were obtained from Charles River, Portage, MI. Hens used in the 1-dose and 5-dose studies were Carey's Nick 320 Leghorns from Carey Farms, Inc., Marion, OH, for comparison of various commercial TCPs; White Leghorns from the Department of Animal and Poultry Science, Virginia Tech, Blacksburg, VA (single-dose NTE screens on low toxicity TCPs); and for the 10-wk study, ISA Brown from Atkinson Bros., Postland, Crowland, Peterborough, England.

Isomer Determinations for Tricresyl Phosphates

GC-MS analyses were performed using a Hewlett-Packard 5890/5972 MSD equipped with a J&W 40 m × 0.182 mm (0.4 mm film thickness) capillary column interfaced directly to the mass spectrometer ion source. The instrumental operating parameters are shown in Table 2.

For TOCP, TMCP, and TPCP, the *ortho* isomer is identified by the relative ratios of the m/z 165 fragment ion (base peak in this case), m/z 368 molecular ion, and the comparatively weak $[M - 1]^+$ ion. Di-*ortho*-cresyl xylyl phosphates have a base peak at m/z 179 (i.e., 165 ± 14) and a very weak $[M - 1]^+$ ion in their spectra. The base peak for di-*ortho*-cresyl ethylphenyl phosphates is at m/z 165, with a very weak $[M - 1]^+$ ion and an intense m/z 179 fragment ion. Determination of *ortho* isomer content of commercial TCPs was based on these ratios.

Chemical Analysis of Phosphate Ester Hydrolysates

Each triaryl phosphate (4 drops) was hydrolyzed with 4 ml of 0.5 *N* methanolic NaOH (Ampoule A, SAP-ESTER kit, Alltech GC Reagent). The

TABLE 1. Inhibition of Rat Blood Serum Cholinesterase (24 h) and Brain NTE (24 h) After a Single Dose (2000 mg/kg) of Various Aryl Phosphates

	Aryl phosphate			Cholinesterase (IU/L) ± SEM			NTE (nmol/min/g) ± SEM		
Class	Supplier/product[a]	Batch	Sample number	Control	Aryl phosphate	Percent inhibition	Control	Aryl phosphate	Percent inhibition
Tricresyl phosphate	1/A	1	86325	491 ± 38 (15)	120 ± 19 (15)	76	344 ± 12 (20)	82 ± 11 (20)	76
		2	88267	441 ± 49 (5)	163 ± 16 (5)	63	374 ± 18 (5)	166 ± 12 (5)	56
		3	88268	530 ± 114 (5)	149 ± 20 (5)	72	374 ± 18 (5)	149 ± 26 (5)	60
		4	88269	504 ± 26 (5)	139 ± 29 (5)	72	374 ± 18 (5)	150 ± 35 (5)	60
		5	88308	544 ± 33 (5)	87 ± 18 (5)	84	341 ± 28 (5)	96 ± 9 (5)	72
		6	88356	575 ± 52 (5)	84 ± 16 (5)	85	341 ± 28 (5)	82 ± 13 (5)	76
		7	89105	510 ± 38 (5)	80 ± 14 (5)	84	407 ± 26 (5)	89 ± 17 (5)	78
		8	92053	459 ± 51 (5)	99 ± 6 (5)	78	916 ± 38 (5)	400 ± 26 (5)	56
		9	92069	603 ± 97 (5)	140 ± 10 (5)	77	916 ± 38 (5)	416 ± 34 (5)	55
	1/B	1	88360	463 ± 17 (10)	78 ± 6 (10)	83	367 ± 18 (10)	217 ± 15 (10)	40
		2	88593	574 ± 104 (5)	114 ± 13 (5)	80	431 ± 5 (5)	314 ± 15 (5)	27
	2/A	1	88213	516 ± 65 (5)	182 ± 11 (5)	65	374 ± 18 (5)	89 ± 15 (5)	76
		2	88311	653 ± 89 (5)	109 ± 23 (5)	83	341 ± 28 (5)	123 ± 38 (5)	64
	3/A	1	88176	561 ± 84 (5)	120 ± 11 (5)	79	326 ± 11 (5)	78 ± 14 (5)	76
	4/A	1	90468	432 ± 30 (10)	92 ± 9 (10)	79	474 ± 18 (10)	403 ± 22 (10)[b]	15
		2	90785	468 ± 37 (5)	72 ± 9 (5)	85	668 ± 28 (5)	570 ± 34 (5)[b]	15
	5/A	1	88720	469 ± 27 (5)	128 ± 12 (5)	73	448 ± 17 (5)	494 ± 14 (5)[b]	0
		2	89127	459 ± 27 (10)	106 ± 12 (10)	77	394 ± 16 (10)	390 ± 12 (10)[b]	1
		3	89656	417 ± 40 (5)	61 ± 5 (5)	85	407 ± 26 (5)	406 ± 48 (5)[b]	0
Cresyldiphenyl phosphate	5/B	1	88211	530 ± 33 (10)	64 ± 4 (10)	88	402 ± 12 (10)	346 ± 19 (10)[b]	14
		2	83309	482 ± 36 (5)	51 ± 9 (5)	89	341 ± 28 (5)	50 ± 20 (5)	85
		3	88310	491 ± 71 (5)	56 ± 5 (5)	88	349 ± 27 (5)	221 ± 20 (5)	37

(Table continues on next page)

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TABLE 1. Inhibition of Rat Blood Serum Cholinesterase (24 h) and Brain NTE (24 h) After a Single Dose (2000 mg/kg) of Various Aryl Phosphates (*Continued*)

Class	Supplier/product[a]	Batch	Sample number	Cholinesterase (IU/L) ± SEM			NTE (nmol/ming) ± SEM		
				Control	Aryl phosphate	Percent inhibition	Control	Aryl phosphate	Percent inhibition
Trixylenyl phosphate	1/C	1	88256	445 ± 38 (5)	112 ± 19 (5)	75	349 ± 27 (5)	71 ± 24 (5)	80
Triaryl phosphate	4/B	1	88671	467 ± 56 (5)	144 ± 33 (5)	69	420 ± 6 (5)	139 ± 34 (5)	67
	4/C	1	90320	549 ± 64 (5)	331 ± 38 (5)	39	350 ± 11 (5)	351 ± 14 (5)[b]	0
	1/D	1	88307	599 ± 80 (50)	85 ± 13 (5)	86	341 ± 28 (5)	112 ± 6 (5)	67
		2	88592	419 ± 64 (5)	104 ± 10 (5)	75	431 ± 5 (5)	40 ± 11 (5)	91
Triisopropylphenyl phosphate	6/A	1	88590	414 ± 47 (5)	80 ± 13 (5)	81	431 ± 5 (5)	211 ± 14 (5)	51
		2	88212	568 ± 53 (5)	54 ± 15 (5)	90	384 ± 16 (5)	156 ± 18 (5)	59
		3	88312	405 ± 43 (5)	43 ± 20 (5)	89	349 ± 27 (5)	139 ± 15 (4)	60
	6/B	1	88589	408 ± 27 (5)	79 ± 5 (5)	81	416 ± 13 (5)	270 ± 16 (5)	35
	3/B	1	88588	320 ± 27 (5)	49 ± 9 (5)	85	416 ± 13 (5)	177 ± 12 (5)	57
		2	91121	457 ± 23 (5)	74 ± 11 (5)	84	668 ± 28 (5)	404 ± 37 (5)	40
	3/C	1	88587	375 ± 39 (5)	61 ± 6 (5)	84	416 ± 13 (5)	194 ± 11 (5)	53
	4/D	1	90469	393 ± 40 (5)	72 ± 9 (5)	81	475 ± 18 (10)	276 ± 24 (10)	42
Butylated triphenyl phosphate	1/E	1	88672	502 ± 26 (5)	530 ± 30 (5)[b]	0	420 ± 6 (5)	365 ± 17 (5)[b]	13
		2	88174	460 ± 65 (10)	270 ± 76 (10)	41	371 ± 18 (10)	308 ± 10 (10)[b]	17
	1/F	1	88168	659 ± 100 (5)	297 ± 38 (5)	58	326 ± 11 (5)	324 ± 9 (5)[b]	1
	1/G	1	88170	576 ± 57 (5)	239 ± 36 (5)	55	374 ± 18 (5)	308 ± 21 (5)[b]	18
	6	1	91114	436 ± 27 (5)	311 ± 31 (5)	29	668 ± 28 (5)	601 ± 31 (5)[b]	10
Tri-*ortho*-cresyl phosphate	Kodak			536 ± 39 (9)	11 ± 2 (10)	98	382 ± 2 (45)	25.4 ± 1.4 (45)	93.4
Tri-*meta*-cresyl phosphate	Kodak			518 ± 49 (10)	72 ± 3 (9)	86	377 ± 18 (4)	338 ± 16 (4)[b]	10
Tri-*para*-cresyl phosphate	Kodak			536 ± 28 (10)	438 ± 29 (10)	18	338 ± 45 (2)	283 ± 44 (2)[b]	16

Note. Figures in parentheses refer to number of rats used.

[a]Suppliers are indicated by numerals (1–7) and different trade-name products for each supplier by letters (A–H). Since product compositions change over time, neither products nor suppliers are identified by name.

[b]Not statistically significant (*p* > .05).

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TABLE 2. GC/MS Operating Parameters for TCP Analyses

Mass spectrometer		Gas chromatograph	
Ionization mode	Electron impact	Oven temperature	150°C
Acquisition mode	Scanning 45–450 amu	Hold time	1 min
Scan speed	1.0 s/scan	Rate	2°C/min
Electron voltage	70 eV	Final temperature	300°C
Multiplier voltage	2300 V	Hold time	10 min
Source temperature	200°C	Injection temperature	300°C
Source pressure	1×10^{-6} torr	Flow rate	He, 45 cm/s
		Injection mode	Split 50:1

test tube was immersed in hot water for approximately 5–10 min with occasional swirling until the TAP was dissolved completely and the resulting solution reduced to about 3 ml. BF$_3$/methanol (5 ml ampoule B, SAP-ESTER Kit, Alltech GC Reagent) was added, and the solution was boiled for about 5 min with occasional swirling until it was reduced to 6 ml. The tube was cooled to room temperature and the milky mixture was transferred to a 60-ml separatory funnel. Five milliliters of water and 20 ml of a saturated aqueous solution of sodium chloride were added and the tube was rinsed with 10 ml methylene chloride (MC), which was added to the separatory funnel. This mixture was extracted 3 times with about 20 ml MC. About three-fourths of the MC was evaporated from the combined extract, which was then dried with anhydrous sodium sulfite.

Trimethylsilyl (TMS) derivatives were made from the phenolic moieties in the MC extract by adding about 0.8 ml extract and 1 ml bis(trimethylsilyl)trifluoroacetamide (BSFTA) (1 ml/ampoule, BSTFA + trimethylchlorosilane (TMCS), 99:1, Sylon BFT kit, Supelco, 3-3148) in a 2-ml GC vial. The vial was capped, and a small syringe needle was inserted through the cap, to avoid a pressure buildup when the vial was placed in an oven at 110–120°C for 15 to 20 min. The vial was then allowed to cool, and the resultant TMS derivatives were analyzed using a Hewlett Packard gas chromatograph, model HP-5890 Series II, equipped with an on-column injector, an automatic sampler (HP-7673), and a flame ionization detector. The instru-

TABLE 3. GC Operating Conditions for Analysis of TCP Hydrolysates

Column	Sicosteel MXT-1, 30 m × 0.53 mm ID, 0.25 μm film thickness (Restek Corporation, Bellefonte, PA)
Oven temperature	20°C to 100°C at 20°C/min, hold 20 min and then 100°C to 350°C at 20°C/min, final time = 3.5 min
Injector	On-column at 35°C to 400°C at 100°C/min, final time = 50 min
Detector temperature	400°C
Solution concentration	As obtained from the hydrolysis and TMS derivatives
Injection size	0.1 μl
Carrier gas	Helium, flow 1.5 ml/min, constant flow

mental operating conditions are shown in Table 3. Phenolic components of most of the TAPs were found to have the same GC response factor (within experimental error). Therefore, the GC peak areas were directly used to determine the relative amounts of all of the phenolic TAP precursors.

Cholinesterase Assays: Phosphate Ester Screening Program

Long-Evans rats were given a single oral dose of 2 g/kg neat phosphate ester or physiological saline for negative controls to provide handling similar to that of phosphate ester-dosed rats. Blood was collected from the orbital sinus just prior to dosing and 24 h later. Serum pseudocholinesterase (S-CHE) activity was determined by the colorimetric method of Ellman et al. (1961). In this assay, propionylthiocholine is hydrolyzed to release thiocholine, which, after further chemical reaction, produces a yellow color (405 nm).

Subchronic Oral Administration of TCP (86325) to the Hen

Each treatment group consisted of 30 hens receiving 2 g/kg of test article, 5 d/wk (Monday–Friday); hens were dosed until the day before sacrifice. Dates of sacrifice were staggered to accommodate the large number of postmortem observations/tests to be performed. Scheduled sacrifice dates were: "6 wk," d 39–44; "8 wk," d 53–54; and "10 wk," d 71–75. Seven dosing groups received test articles of known total organophosphate content: (1) jet engine oil (JEO) minus TCP or TOCP, (2) JEO + 3% TCP, (3) JEO + 1% TCP, (4) JEO + 0.5% TCP, (5) JEO + 0.5% TOCP, (6) corn oil (CO) + 0.5% TOCP, and (7) untreated controls. The experimental design was previously described (Freudenthal et al., 1993).

Cholinesterase Assays: Subchronic Hen Study

In this study, pseudocholinesterase determinations were conducted by the Ellman et al. (1961) procedure, but on plasma rather than serum, as specified by the SOP of the testing laboratory. This change is thought to have no effect on the degree of inhibition observed. Brain homogenates were also assayed for true or acetylcholinesterase by the same colorimetric procedure. Comparisons were made with a separate control group, rather than having each animal serving as its own control.

NTE Assay: Phosphate Ester Screening Program and 1- Versus 5-Day Hen Study

NTE determinations were performed according to the original method of Johnson (1977), with slight variations. For screening neat phosphate esters, the same rats used for the S-CHE assays were sacrificed at 44 h after dosing. Whole brains were removed, chilled, and homogenized using a Polytron. Homogenates were centrifuged at 9000 × g for 15 min. NTE activity was determined on the supernatant with reagents obtained from Chem Science Laboratories and according to their protocol. Paired 100-μl aliquots

of the supernatant were preincubated with paraoxon in one tube and paraoxon plus mipafox in another. After 20 min, phenyl valerate was added to both tubes as a substrate for hydrolysis by the remaining uninhibited enzyme. Hydrolysis was stopped by the addition of sodium dodecyl sulfate, and color developed by the reaction of valeric acid with 4-aminoantipyrine and potassium ferricyanide. The difference in absorbance at 510 nm between the tubes in each pair corresponds to the amount of NTE activity in that particular brain. Results are reported as nanomoles phenyl valerate metabolized per minute per gram of whole brain, wet weight, and as percent inhibition compared to a control group.

NTE Assay: Subchronic Hen Study

The experimental design for this study has been described previously (Freudenthal et al., 1993). NTE assays were performed on whole brain and spinal cord homogenates from hens sacrificed 24 h after the final dose in a 6-, 8-, or 10-wk treatment period. Brains and spinal cords were frozen using dry ice/hexane and stored at –20°C for 27 to 40 d before assay. In this study, assays were performed on whole homogenates. Data were reported as before.

NTE Assay: Single-Dose Hen Studies
for Comparison of Various Commercial TCPs

Hens were sacrificed 24 h after oral dosing at 2 g/kg. Whole brains and spinal cords were stored at –70°C until assayed. The standard NTE assay was adapted to be run in a microtitre plate at 1/20th of the usual assay volume (Correll & Ehrich, 1991). Concentrations of all reagents were equivalent to the other NTE assay procedures used. In these studies, NTE activity is reported as nanomoles substrate hydrolyzed per minute per milligram protein, rather than per gram whole brain, wet weight. Protein determinations were performed using a Bio-Rad kit-based Coomassie brilliant blue microassay.

Grading for Ataxia: Subchronic Hen Study

Each hen was evaluated daily outside the cage, while walking, running, and jumping. Abnormalities in gait were scored on an 8-point scale according to the system developed by Cavanagh et al. (1961). A score of 8 represents complete inability to stand or walk.

Histopathological Examinations: Subchronic Hen Study

At sacrifice, birds scheduled for histopathological examination were perfused with 10% neutral buffered formalin; the spinal column was then removed and stored in the same fixative. One transverse section and two longitudinal sections were taken at each of four spinal cord levels: upper cervical, lower cervical, thoracic, and lumbar. These sections were processed and embedded in paraffin wax. Sections were cut at 7 µm and

stained with hematoxylin and eosin. Additional sections from each block were stained according to the method of Marsland et al. (1954) for axons, and with solochrome cyanin (Page, 1970) for myelin. Degenerative effects were scored on a scale from I to V: I, no white-matter abnormality; II, occasional fragmentation of axons; III, disruption, fragmentation, or distortion of a few axons; IV, disruption, fragmentation, or distortion of moderate numbers of axons, with disruption of associated myelin sheaths; and V, disruption, fragmentation, or distortion of many axons. Grades III and above indicate serious degeneration. An overall grade was assigned to each slide (two longitudinal and one transverse section/slide).

RESULTS

Screening of Triaryl Phosphates: Inhibition of Rat Brain NTE (B-NTE) and Serum Cholinesterase (S-CHE)

Six TAP types, distinguished by their phenol constituents, were selected for evaluation (Table 1). Products from each class were obtained from suppliers that Mobil has been using or might consider using in the future. In addition, for some of the products, multiple production batches were screened. Product and supplier names were coded to maintain confidentiality since the products are complex mixtures, and the composition of current production may differ from that tested. Pure TOCP, TPCP, and TMCP were also tested as controls.

Triaryl phosphates were screened at a single oral dose of 2 g/kg in the rat for inhibition of S-CHE (24 h post dose) and B-NTE (44 h post dose). With the exception of a single butylated triphenyl phosphate (number 88672), all of the TAPs significantly inhibited S-CHE. Tri-*para*-cresyl phosphate produced only a slight inhibition (18%). In general, the butylated triphenyl phosphates produced lower levels of S-CHE and B-NTE inhibition than did the other classes.

B-NTE inhibition varied widely between and within classes and from batch to batch. For example, inhibition by TCP ranged from a high of 78% (89105) to 0% (88720, 89656); the other TAPs ranged from 91% (88592) to 0% (90320). Butylated triphenyl phosphates did not significantly inhibit B-NTE, and neither TMCP nor TPCP showed significant inhibition. Tri-*ortho*-cresyl phosphate (TOCP) produced a 93.4% inhibition, and thus was more potent than any of the triaryl phosphate products tested.

With all but three TAPs of Table 1 (88213, 88256, 88592), S-CHE was inhibited to a degree equal to or greater than that for B-NTE, supporting use of S-CHE inhibition as a marker of acute exposure and therefore potential neurotoxicity of TAPs. Lack of significant S-CHE inhibition after an exposure incident would suggest that a neurotoxic outcome would not occur.

For screening purposes, a statistically significant inhibition of B-NTE in the rat (corresponding to an inhibition of ~20%) was considered to indicate that a triaryl phosphate might possibly cause OPIDN if an equal

dose were given to hens. The Long-Evans rat does not develop OPIDN until B-NTE inhibition approaches ~70% (Padilla & Veronesi, 1985), but the hen is considerably more sensitive than the rat and will develop this level of NTE inhibition and OPIDN at a lower dose (Ehrich et al., 1995). For a given dose, 20% inhibition in the rat corresponds to approximately 70% in the hen (later discussion). Most of the TAPs of Table 1, with the exception of the butylated triphenyl phosphates and several other specific TAP products, would be predicted by the screen to be capable of causing OPIDN in a more sensitive species (e.g., the hen) at an appropriate dose level. Chemical analysis showed that *ortho*-cresyl groups accounted for only 0.36 to 2.38% of the phenolic moieties in sample hydrolysates for TCPs that inhibited rat B-NTE by 27 to 76%.

Hen and Rat B-NTE Inhibition After Acute Oral Doses of TCP

At a given oral dose of TOCP (as mg/kg body weight), hen B-NTE (Dudek, 1979) is inhibited to a greater degree than rat B-NTE (Padilla & Veronesi, 1985; Ehrich et al., 1995). TCP 86325 was employed as our standard inhibitor in the rat. Figure 1 shows the inhibition of rat and hen B-NTE by single doses of TCP or TOCP. Dose response curves were linearized by probit transformation. The equations of lines A–C are $y = 1.64x + 2.56$, $y = 1.67x + 1.82$, and $1.71x - 0.01$, respectively. The consistency of the slopes permits calculation of potency (or sensitivity) ratios between species and between different TCPs (including TOCP) at any level of NTE inhibition. In the rat, TCP (86325) was 0.34 times as potent as TOCP. It was found that TOCP was less potent in the hen than reported by Dudek (1979). From lines A and B the 70% inhibition level in the hen, which is the approximate level at which OPIDN is expected to occur, was reached at 164 mg/kg in our study and in the Dudek (1979) study at 63.8 mg/kg. TOCP data from Figure 1 permit the evaluation of the species sensitivity ratio, the quotient of the dose rates at which two different species exhibit the same effect in response to the same test material. At 93% B-NTE inhibition, the rat:hen ratio is 8.35 using the Dudek data (line A) and 3.25 using the Mobil data (line B). Because of the near-parallelism of the slopes, these ratios change little over the range from 20% to 95% inhibition. Statistical significance for rat B-NTE inhibition is achieved at approximately 20% and higher (Table 1). This corresponds to hen B-NTE inhibition between 70% (line A) and 43% (line B). Thus, a sample producing about 20% B-NTE inhibition in the rat at 2 g/kg might, at the same dose, inhibit NTE in the hen to a level that could cause OPIDN. TCPs showing no indication of inhibition in the rat screen would be unlikely to cause 70% B-NTE inhibition and OPIDN in 2-g/kg single-dose hen assay.

Screening of Tricresylphosphates: Inhibition of Hen B-NTE

Several tricresyl phosphates, prepared from phenols with very low levels of *ortho* substitution, were screened directly in the hen rather than
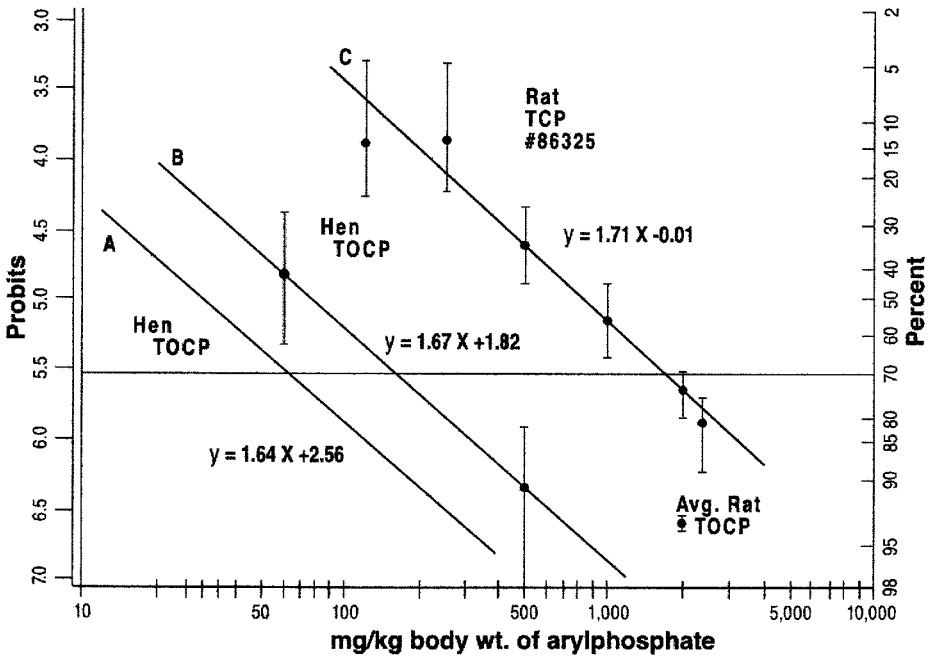
FIGURE 1. Dose-response curves for brain NTE inhibition after oral administration of TCPs to rat and hen. Line A: Hen brain NTE inhibition data (24 h) from Dudek (1979) dose-response study. Line A represents a log-probit transformation of NTE inhibition for doses of 5 to 65 mg/kg. Line B: Hen brain NTE inhibition data (24 h) from TOCP positive controls for screening studies on low-*ortho* TCPs. Line C: Rat brain NTE inhibition (44 h), dose-response study. The single point at the lower right is the average rat brain NTE inhibition for the TOCP positive control, administered at 2000 mg/kg, in each of the TAP screening studies. Each data point for lines A–C represents the mean ± SEM of four replicates. The data point for TOCP in the rat is from Table 1.

the rat to assure an adequate level of test sensitivity. Results are shown in Table 4. Two separate experiments, A and B, were performed sequentially, and TCP 96297 was evaluated in both experiments. Hens received a single oral dose (2 g/kg), and brain tissue for NTE measurement was taken 24 h later, the standard time for NTE assays in this species. A low, but detectable, level of inhibition was seen with all TCP samples; results with 96297 did not reach statistical significance, however. These TCPs were found by chemical analysis to have only about 10% of the amount of *ortho*-cresyl substituents present in commercial TCPs manufactured in the 1980s. An even greater decrease was seen for *ortho*-xylyl substituents. It is unlikely that any of these TCPs would elicit a neuropathological response, considering the very high dose used in this sensitive species and the low level of B-NTE inhibition observed. S-CHE was not evaluated in these experiments, since inhibition would have been expected in a manner similar to that seen for the other TCPs of Table 1.

TABLE 4. Inhibition of Hen Brain NTE (24 h) After a Singular 2 g/kg Dose of Low-*Ortho*-Tricresylphosphate

Experiment	TCP Supplier/ product	Batch	Sample	B-NTE activity (nmol/min/mg protein) ± SEM Control[a]	TCP[a]	Percent inhibition
A	5/A	4	96297	15.88 ± 0 64 (6)	11.40 ± 0.53 (6)	28
	1/H	1	96298	15.88 ± 0.64 (6)	11.21 ± 1.84 (6)	29
	4/E	1	96299	15.88 ± 0.64 (6)	10 51 ± 1.30 (6)	34
	5/A	5	96300	15.88 ± 0 64 (6)	11.88 ± 1.00 (6)	25
B	5/A	4	96297	25.67 ± 2 45 (6)	20.68 ± 1.52 (6)	19
	1/H	2	97002	25.67 ± 2.45 (6)	16.75 ± 1.42 (6)	35
	5/A	6	97003	25.67 ± 2.45 (6)	15.60 ± 1.36 (6)	39
	7	1	97004	25.67 ± 2.45 (6)	16.33 ± 1.66 (6)	36

Note. See footnote *a* for Table 1. TCPs were prepared from phenols with very low level of *ortho* substitutions.

[a]Number in parentheses is number of hens on test.

Subacute Testing of TCP and TOCP in the Rat and Hen

Corn oil (CO) and jet engine oil (JEO) + 3% TCP (86325) or TOCP were evaluated in rat and hen to determine if cumulative inhibition of B-NTE would occur and to evaluate whether the potential toxicity of TCP in JEO might be reduced by retarding intestinal adsorption. Table 5 summarizes the results of this study. Rats and hens received 2 g/kg/d doses of JEO + 3%

TABLE 5. Inhibition of B-NTE in Rat and Hen After Subacute (5-day) Oral Administration of Oils Containing TCP or TOCP[a]

Species	Test article (2000 mg/kg/d)	Phosphate ester dose (mg/kg/d)	(mg/kg/5 d)	B-NTE Activity ± SEM (nmol/g)	Inhibition[a] (%)	Incidence of ataxia
Rat	CO	0	0	484 ± 14	0	—
	JEO + 3% TCP	60	300	460 ± 9.4[c]	5	0/5
	CO + 3% TOCP	60	300	379 ± 26[b]	22	0/5
	JEO + 3% TOCP	60	300	429 ± 18[b]	11	0/5
Hen	CO	0	0	1212 ± 112	0	—
	JEO + 3% TCP	60	300	819 ± 74[b,c]	32	0/4
	CO + 3% TOCP	60	300	162 ± 20[b]	67	2/4
	JEO + 3% TOCP	60	300	240 ± 36[b]	80	2/4

[a]Animals (5 rats or 4 hens/group) received 2000 mg/kg/d of test article by oral gavage. TCP was #86325.

[b]Significantly different from control (CO). $p < .05$.

[c]Significantly different from CO + TOCP and JEO + TOCP. $p < .05$.

TCP or TOCP, and of CO + 3% TOCP, for 5 consecutive days and were then sacrificed for determination of B-NTE activity. TOCP, whether administered at 3% in CO or JEO, produced significant inhibition of B-NTE in the rat and a greater inhibition in the hen. TOCP showed slightly greater B-NTE inhibition when administered in CO than in JEO, but the difference was not statistically significant. There did not appear to be a substantial cumulative effect at these doses in the rat over the acute effects shown in Figure 1, but there were obvious cumulative effects with both TCP and TOCP in the hen. The hens receiving the 5 doses of CO + TOCP and JEO + TOCP developed ataxia during a 30-d postdose observation period (Barth et al., 1993).

Since NTE inhibition is irreversible, and recovery of activity occurs through the prolonged process of enzyme synthesis, it is likely that in the rat metabolic clearance prevents cumulative inhibition after multiple low-dose administrations. In accord with this, it has been shown that after a given dose of TOCP, concentration in plasma of both TOCP and its active metabolite saligenin-*o*-cresyl phosphate were much lower in the rat, and the half-life of active metabolite in the rat was about one-third that in the hen (Suwita & Abou-Donia, 1990). The sensitivity of the rat assay is therefore not enhanced by repeated dosing. In the hen, a single 60-mg/kg dose of TCP also did not cause a detectable B-NTE effect, but multiple dosing produced a statistically significant 32% inhibition. In this species, repeated dosing increases the ability to detect inhibitory activity. TCP (86325) is known to produce OPIDN when present in a JEO at 3% and administered to hens at 2 g/kg/d for 10 wk (see later discussion and Freudenthal et al., 1993). Thus, neither the rat acute nor hen subacute B-NTE screens could be expected to detect weak or moderately active TCPs at low concentrations in a formulated lubricant, unless very high doses (e.g., more than 2 g/kg) are administered (Barth et al., 1993).

Subchronic Testing of TCP (86325) in Jet Engine Oil

JEOs containing TCP have been evaluated in a hen 10-wk oral bioassay to evaluate the potential to cause OPIDN and inhibit B- and spinal cord (SC-) NTE as well as plasma (P-) and B-CHE. A portion of the results was previously published by Freudenthal et al. (1993). Oils containing 0.5–3.0% TCP or 0.5% TOCP in either JEO or CO were administered orally at 2 g/kg/d. Hens were observed for symptoms of neurotoxicity daily and were sacrificed at 6, 8, or 10 wk for analyses of B- and SC-NTE, B- and P-CHE, and brain and spinal cord histopathology. Enzymological results are shown in Table 6. All of the enzyme activities were inhibited by the oils containing TCP and TOCP. P-CHE was inhibited by approximately 35% and B-CHE by about 14% in a non-dose-responsive manner confirming the previous observations of similar effects seen in the rat after subchronic oral administration of TCP (86325) (see earlier footnote). There were significant correlations between degree of P-CHE inhibition

TABLE 6. Inhibition of Cholinesterase (CHE) and Neuropathy Target Esterase (NTE) in Hens After Subchronic Oral Administration of Oils Containing TCP or TOCP

Test group	Test article (2000 mg/kg/d)	n^a	Percent inhibition ± SE			
			P-CHE	B-CHE	SC-NTE	B-NTE
1	JEO – TCP (or TOCP)	15	—	—	—	—
4	JEO + 0.5% TCP	15	29.4 ± 5.2	13.9 ± 5.4	29.9 ± 6.0	46.4 ± 3.0
3	JEO + 1.0% TCP	14	28.8 ± 5.5	11.1 ± 4.0	39.9 ± 5.5	52.6 ± 3.2
2	JEO + 3% TCP	13	47.7 ± 5.2	11.0 ± 5.0	62.2 ± 4.6	77.9 ± 2.6
5	CO + 0.5% TOCP	15	45.1 ± 5.6	15.3 ± 4.6	53.6 ± 3.4	68.4 ± 2.2
5	JEO + 0.5% TOCP	15	35.3 ± 4.2	14.9 ± 4.0	52.3 ± 4.6	63.4 ± 3.7
8^b	Untreated controls	5	—	—	—	—

Note. JEO-TCP (Group 1) was used to prepare the test articles for Groups 2–5. Values for enzyme inhibition are mean differences (%) ± SEM from Group 1. Data from determinations at 6, 8, and 10 wk of exposure were combined since inhibition was the same at each sacrifice and there was no trend over time. There were no significant differences in enzyme activities between Groups 1 and 8.

^{a}Number of hens.
^{b}Group 8 hens were evaluated for enzyme inhibition only at wk 10.

and inhibition of both SC-NTE and B-NTE ($r = .94$, $p = .02$). However, this is not an indication that cholinesterase inhibition is, in general, correlated with NTE inhibition. As shown in Table 1, TOCP was a more potent inhibitor of both NTE and CHE than were any of the TCPs that were available for our screening program. For the TCP class in general (Table 1), CHE inhibition was not correlated with NTE inhibition. B-NTE and SC-NTE were inhibited by TCP in a dose-responsive manner, and by TOCP in both JEO and CO. There was no statistically significant difference in enzyme activity between TOCP in JEO and CO. With both TCP and TOCP, B-NTE was inhibited to a greater degree than was SC-NTE, and the standard errors for B-NTE inhibition were lower than for SC-NTE inhibition, indicating that B-NTE inhibition would be a more reliable and more sensitive indicator of neurotoxic potential than measurement of SC-NTE. The dose-response curves (not shown) for inhibition of both SC-NTE and B-NTE versus daily dose (mg/kg) of the TCP administered in JEO were linear. The equations for the TCP dose versus SC-NTE and B-NTE lines were $y = 0.625x + 25.2$ and $y = 0.630x + 40.0$, respectively.

Only the JEO + 3% TCP and CO + 0.5% TOCP elicited OPIDN as indicated by paralytic symptoms and concomitant spinal cord pathology. With JEO + 3% TCP, mean inhibition of B- and SC-NTE was 77.9% and 62.2%, respectively, and for CO + 0.5% TOCP, 68.4% and 53.6%, respectively (Table 6). Of the 30 hens receiving JEO + 3% TCP, 22 (73%) became ataxic (average severity grade 4.3); of the hens receiving CO + 0.5% TOCP, 3 of 30 (10%) became ataxic (average severity grade 3.0) (Freudenthal et al., 1993). Thus, ataxia occurred only in the dosing groups with the highest levels of B- and SC-NTE inhibition.

The time course for the development of ataxia is shown in Table 7. For birds given JEO + 3% TCP (60 mg/kg TCP), ataxia was generally first observed between 16 and 35 d after dosing began, although 3 birds did not exhibit symptoms until much later. Onset of ataxia was, on the average, later for birds given CO + 0.5% TOCP (10 mg/kg TOCP). The most severe cases of ataxia and paralysis were associated with earlier onset.

The 0.5% TOCP dose in either CO or JEO produced mean B- and SC-NTE inhibitions that were not significantly different from each other, but there was increased toxicological activity when TOCP was administered in CO, as indicated by observation of paralytic symptoms in three hens (group 7, Table 7). It is likely that the uptake of TOCP was enhanced by dissolution in CO, since CO is entirely absorbed and metabolized, whereas

TABLE 7. Ataxia Scores for Hens Subchronically Exposed by Oral Gavage to a Generic Jet Engine Oil Containing 3% TCP (86325) or to Corn Oil Containing 0.5% TOCP
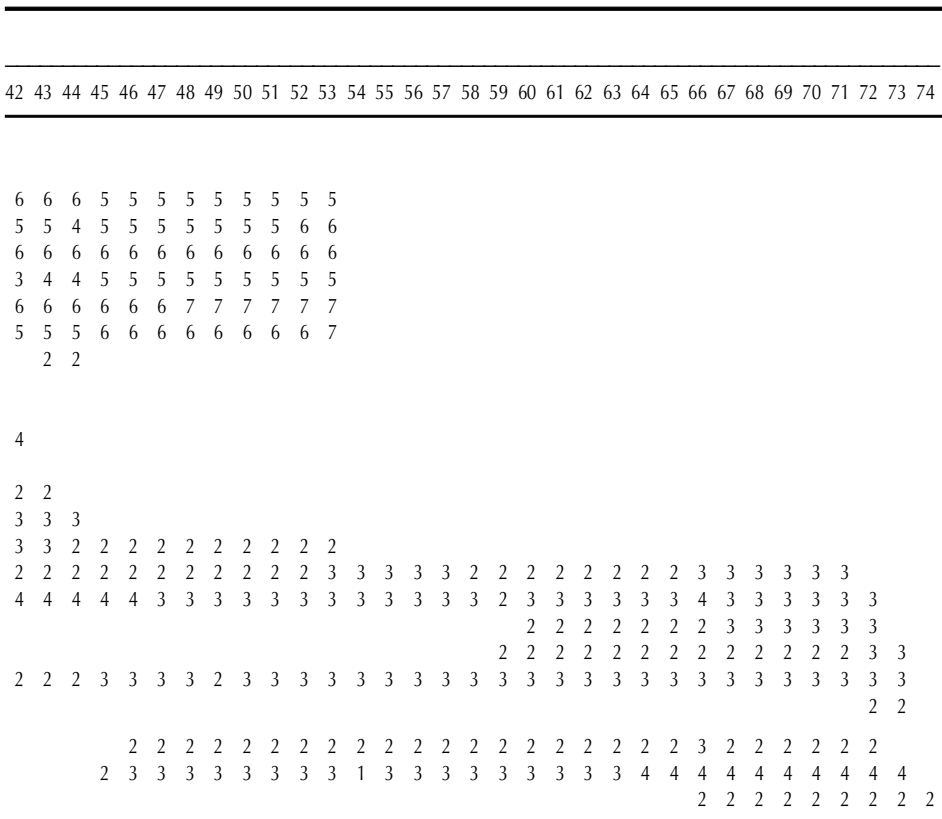
Group	Bird number	1–15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
2	32									2	2	4	4	4	5	5	5	5	6	6	6	6	6	6	5	5		
	33																2	2	3	3	2	2	2	2	1	1		
	36									2	2	2	3	3	4	4	5	5	6	6	5	6	6	6	5	5	5	5
	37										2	2	2	2	2	2	2	2	3	3	2	4	4	4	4	4	4	4
	38								2	4	4	6	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	39										2	2	2	2	3	2	3	3	4	4	3	4	4	4	4	4	4	4
	40									2	2	2	3	2	2	2	3	4	4	5	5	5	6	6	6	6	6	6
	1604											1	2	2	2	2	2	2	2	4	3	5	5	5	5	5	5	5
	43																											
	44		2	2	3	3	3	4	5	6	6	6	6	7	7													
	45																					2						
	47									2	2	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	48			2	2	2	2	3	4	5	5	6	6	7	7													
	49															2	2	2	2	3	3	2	2	2	2	2	3	3
	50																2	2	2	2	2	3	3	3	3	3	3	3
	53																					2	2	3	2	2	2	2
	54											1	1	0	2	2	2	2	2	2	3	2	2	2	2	2		
	56									2	2	2	2	2	2	2	2	3	3	4	4	3	4	3	3	3	3	3
	57																					2						
	58																											
	59														2	2	2	2	2	2	2	2	2	2	2	2	2	2
	60																					2						
7	205																											
	207																											
	209																											

Note. Ataxia scores are given for each hen from day of onset until sacrifice or unscheduled death. Oils were administered at 2000 mg/kg by oral gavage. Scheduled sacrifices were on d 39, 42, 43, 44, 53, 71, 72, 73, and 74. Group 2 hens received jet engine oil containing 3% TCP and Group 7 hens received corn oil containing 0.5% TOCP. No other hens in Groups 1–8 showed any indication of an ataxic effect; except for sacrificed birds, blank

most of the JEO passes entirely through the gastrointestional (GI) tract, and therefore would be expected to retain a portion of the hydrophobic TOCP. The small difference in TOCP absorption, as indicated by the enzymological findings, suggests that for the affected hens, a critical threshold of NTE inhibition was reached and that this culminated in the observed symptoms of OPIDN.

The existence of such a threshold can be observed by examining the relationship between inhibition of NTE in brain and spinal cord and the development of ataxia. Figure 2 shows the ataxia scores and levels of NTE inhibition (for all hens in which these parameters were measured) in the groups receiving either TCP or TOCP. The hens were sacrificed at 6, 8, or 10 wk of exposure. Figure 2a relates ataxia to B-NTE inhibition and Figure

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42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74


6  6  6  5  5  5  5  5  5  5  5  5
5  5  4  5  5  5  5  5  5  5  6  6
6  6  6  6  6  6  6  6  6  6  6  6
3  4  4  5  5  5  5  5  5  5  5  5
6  6  6  6  6  6  7  7  7  7  7  7
5  5  5  6  6  6  6  6  6  6  6  7
      2  2


4


2  2
3  3  3
3  3  2  2  2  2  2  2  2  2  2  2
2  2  2  2  2  2  2  2  2  2  2  3  3  3  3  3  2  2  2  2  2  2  2  2  3  3  3  3  3  3
4  4  4  4  4  3  3  3  3  3  3  3  3  3  3  3  3  3  2  3  3  3  3  3  3  4  3  3  3  3  3  3
                                                      2  2  2  2  2  2  2  2  3  3  3  3  3  3
                                                         2  2  2  2  2  2  2  2  2  2  2  2  2  3  3
2  2  2  3  3  3  3  2  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3  3
                                                                                        2  2

            2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  3  2  2  2  2  2  2
         2  3  3  3  3  3  3  3  3  3  1  3  3  3  3  3  3  3  3  3  3  4  4  4  4  4  4  4  4  4
                                                            2  2  2  2  2  2  2  2  2
```

spaces indicate a score of zero. Key for scoring ataxia: 0, no ataxia; 2, slight incoordination; 3, frequent incoordination; 4, staggering gait; 5, continuous staggering gait, tail and leg reflexes usually noticeably affected; 6, bird stands for short periods only, moves by shuffling on hocks; 7, as in 6, reflexes markedly affected; 8, bird unable to stand, weak limb movements, reflexes virtually nonexistent.
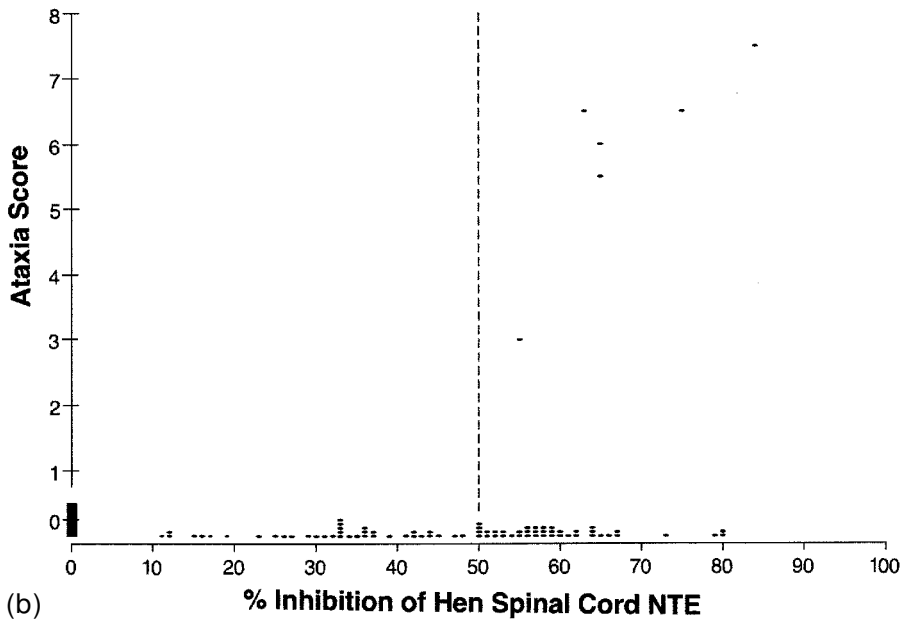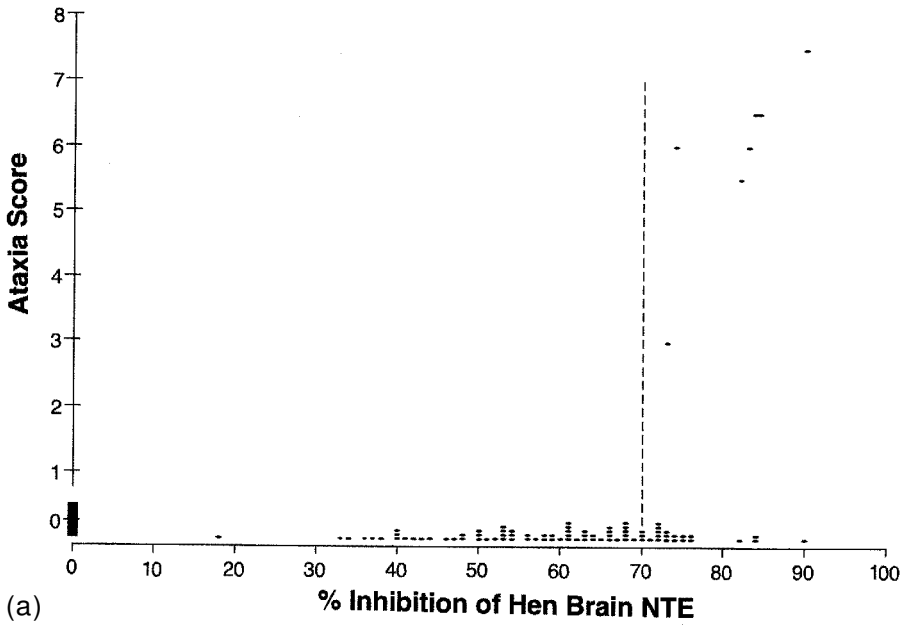
(a)

(b)

FIGURE 2. Ataxia as a function of NTE inhibition for all birds from the 10-wk hen study receiving TCP or TOCP for which NTE was determined. Individual ataxia scores are plotted against (a) B-NTE and (b) SC-NTE inhibition. Birds were sacrificed after 6, 8, or 10 wk of dosing. Vertical lines represent approximate thresholds for development of ataxia.

2b to SC-NTE inhibition. Ataxia was observed only at levels of B-NTE inhibition above 72% (approximate threshold 70%) and SC-NTE inhibition above 54% (approximate threshold 50%). Not all hens with NTE inhibited more than the threshold values exhibited ataxia at time of sacrifice, but it is likely that symptoms would have become apparent in these birds if the sacrifice times had been delayed.

Histopathological effects caused by TCPs result in the subsequent or simultaneous development of the neuropathic symptoms of OPIDN; therefore, it is important to determine if microscopic lesions occur at dose levels (and levels of NTE inhibition) substantially below those that cause ataxia. Table 8 shows the relationship between NTE inhibition, spinal cord neuropathology, and incidence of ataxia. At both the 6- and 10-wk sacrifices, severity of neuropathology and incidence of ataxia were correlated with the levels of SC- and B-NTE inhibition. After 6 wk of dosing, grade III neuropathology was seen at SC- and B-NTE inhibition of 52.3% and 63.4%, respectively (Group 5); however, ataxia was not seen until the levels of inhibition reached 62.2% and 77.9%, respectively (Group 2). After 10 wk of dosing, grade III neuropathology was seen at SC- and B-NTE inhibition of 39.9% and 52.6%, respectively (Group 3); however, ataxia was not seen until the levels of inhibition reached 53.6% and 68.4%, respectively. A threshold-type response relative to NTE inhibition is apparent, and there is a close correspondence between incidence and severity of histopathology, ataxia, and NTE inhibition. The minimally ataxic doses of TCP at 6 and 10 wk were only about 2 times the minimally neuropathic doses.

The levels of SC- and B-NTE inhibition were maximal by the 6-wk sacrifice period, but neuropathology and incidence of ataxia worsened over the following 4-wk dosing period. Apparently, the lesions caused by NTE inhibition develop slowly. It is possible that the progression of neuropathology would have continued even if dosing had been discontinued at 6 wk.

It is clear from results presented in Table 8 that NTE inhibition was the most sensitive indicator of neurotoxic potential. At the 6-wk sacrifice, JEO + 0.5% and 1.0% TCP produced significant levels of inhibition without evidence of serious nerve degeneration (grade III or above) or ataxia. Neuropathology, although a less sensitive parameter than NTE inhibition, was more sensitive than ataxia for predicting OPIDN.

Detection of Potential Neurotoxicity of TCPs in Formulated Oil

The likelihood of a TCP being capable of causing OPIDN can be reasonably estimated by performing acute (single oral dose) studies with the neat material using either B-NTE or development of ataxia as the measured endpoint. However, a fully formulated oil requires dosing for a longer period, since there are limitations on the volume that can be administered in a bolus dose, and there is potential for reduced bioavailability when an inordinately large dose is given.

TABLE 8. Relationship Between NTE Inhibition, Severity of Spinal Cord Histopahtology, and Incidence of Ataxia (OPIDN) for Subchronic Administration of Oils Containing TCP or TOCP

Test group rank[a]	Test article	TOCP as TCP dose equivalent[b]	n	NTE inhibition[c] (%) ± SE SC-NTE	B-NTE	n	Spinal cord neuropathology grade (% of readings)[d] I + II	III	IV	III + IV	Hens with ataxia incidence (%)[e] At	Through
6-wk sacrifice											At 6 wk	Through 6 wk
1	JEO – TCP (or TOCP)	—	15	0	0	5	100	—	—	0	0/30 (0)	0/30 (0)
4	JEO + 0.5% TCP	—	15	29.9 ± 6.0	46.4 ± 3.0	4	100	—	—	0	0/29 (0)	0/30 (0)
3	JEO + 1.0% TCP	—	14	39.9 ± 5.5	52.6 ± 3.2	6	100	—	—	0	0/29 (0)	0/30 (0)
5	JEO + 0.5% TOCP	JEO + 1.86 TCP	15	52.3 ± 4.6	63.4 ± 3.7	5	90	10	—	10	0/39 (0)	0/30 (0)
7	CO + 0.5% TOCP	CO + 2.27 TCP	15	53.6 ± 3.4	68.4 ± 2.2	5	70	30	—	30	0/30 (0)	0/30 (0)
2	JEO + 3.0% TCP	—	13	62.2 ± 4.6	77.9 ± 2.6	5	30	55	15	70	16/28 (57)	21/30 (70)
10-wk sacrifice											At 10 wk	Through 10 wk
1	JEO – TCP (or TOCP)	—	15	0	0	10	100	—	—	0	0/15 (0)	0/30 (0)
4	JEO + 0.5% TCP	—	15	29.9 ± 6.0	46.4 ± 3.0	10	100	—	—	0	0/15 (0)	0/30 (0)
3	JEO + 1.0% TCP	—	14	39.9 ± 5.5	52.6 ± 3.2	9	97	3	—	3	0/13 (0)	0/30 (0)
5	JEO + 0.5% TOCP	JEO + 1.86 TCP	15	52.3 ± 4.6	63.4 ± 3.7	10	80	15	5	20	0/15 (0)	0/30 (0)
7	CO + 0.5% TOCP	CO + 2.27 TCP	15	53.6 ± 3.4	68.4 ± 2.2	10	50	38	12	50	3/15 (20)	3/30 (10)
2	JEO + 3.0% TCP	—	13	62.2 ± 4.6	77.9 ± 2.6	11	32	41	27	68	6/12 (50)	22/30 (73)

[a]Groups are ranked according to inhibition of B-NTE.

[b]The TOCP concentrations were converted to TCP equivalent concentrations by using the TCP dose-response curves for inhibition of SC-NTE and B-NTE.

[c]NTE inhibition is from Table 6.

[d]The % of readings refers to the percent of all scores obtained for each grade on all slides (e.g., for Group 1, there were 40 individual scores for the 10 hens); n is the number of hens sacrificed/group. Serious nerve degeneration occurs at levels III and above. Grades I and II were combined as background. Grades III and IV were also combined to give an estimate of percent of hens with serious degenerative effects. None of the slides in this study received a grade of V.

[e]Data are presented as number of ataxic hens/number of hens in the group and as percent. The "at" column uses the number of hens alive at sacrifice as the number of hens in each group; the "through" column uses the total number of hens entered into the study (30) as the number of hens in each group.

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Comparison of our acute, subacute, and subchronic hen studies for a simulated JEO + 3% TCP or TOCP is shown in Table 9. These results clearly demonstrate that the oil that caused OPIDN upon multiple administration would not have been detected as a possible neurotoxicant in the acute dosing regime, and, furthermore, only the TOCP-containing oil would have shown a 70% or greater inhibition of B-NTE and OPIDN in the subacute 5-d procedure. It is necessary to perform the subchronic test at the high oral dose of 2 g/kg/d for a formulated JEO to detect the minimal dose needed to produce a greater than threshold B-NTE inhibition (6 wk of dosing) or to cause OPIDN itself (10 wk of dosing). The U.S. Environmental Protection Agency (EPA) has recent issued Health Effects Test Guidelines (OPPTS 870.6100) for Acute and 28-Day Delayed Neurotoxicity of Organophosphorus Substances. In the acute test, levels greater than 2 g/kg and in the 28-day study levels greater than 1 g/kg need not be tested. These dose limitations appropriately apply to neat TAPs, but should not be used to evaluate JEO or other formulated oils in which the TAP has been diluted by inert components. With JEO + 3.0% TCP, the TCP is already diluted 33-fold.

The effects of repeated dosing will be cumulative, but for each dose a plateau of effect is expected such that continuous dosing at some levels will be tolerated without causing OPIDN. For each TCP one could determine the dose that would be tolerated in a particular dosing regimen. For TOCP in the hen, an acute dose of less than 164 mg/kg by our results [or 64 mg/kg from the Dudek (1979) dose-response line of Figure 1] would be unlikely to cause OPIDN; in a 5-d subacute study, it would be 60 mg/kg/d, and in a 10-wk subchronic study, it would be less than 10 mg/kg/d.

Chemical Composition of TCP Versus Neurotoxic Potential

As indicated by the results summarized in Table 1, there is significant variability in B-NTE inhibitory activity when comparing TCP samples

TABLE 9. Comparison of Acute, Subacute, and Subchronic Hen Studies for Generic Jet Engine Oils Formulated With Phosphate Esters (PE) Administered at 2 g/kg/d for 1, 5, or 50 Consecutive Days

PE test material	Days of dosing	Daily dose[a]	Total dose (mg/kg)[a]	B-NTE inhibition (%)	Ataxia
TCP	1	60	60	4	No
(86325)	5	60	300	32	No
	50	60	3000	77	Yes
TOCP	1	500	500	91	Yes
	1	60	60	42	No
	5	60	300	80	Yes
	50	10	500	45	No

[a]Phosphate ester only.

from different suppliers and different batches from the same supplier. Because of the nature of the dose-response curves, linearized in Figure 1, difference in potency on a mg/kg basis is larger than is obvious from observing only the percent difference in B-NTE activity. The differences in activity for TCP are determined by phenol and the alkylphenols present in the mixture used for TCP synthesis. For at least 40 yr, it has been recognized that alkyl substitutents at the *ortho* positions of the aromatic rings are responsible for the neurotoxic activity of TCP (Henschler, 1958). Mono-*ortho*-TCP has about 10 times the activity of TOCP and di-*ortho*-TCP about 5 times the activity of TOCP (Henschler, 1958). TCPs made during the 1940s and early 1950s were from high-*ortho*-substituted (25 to 40%) mixtures of phenols and were more neurotoxic than pure TOCP (Henschler, 1958). Since the mid-1950s, manufacturers have been reducing the content of *ortho* substituents, to decrease toxicity. Nevertheless, numerous outbreaks of OPIDN have occurred in humans, almost always after accidental or intentional ingestion, from the TCPs with lowered *ortho* content. Since the U.S. EPA TSCA Section 8e filing by Mobil Oil Corporation in 1988, which reported potential neurotoxicity of a contemporary TCP being used in jet engine oil, there have been further reductions in the *ortho* substituents.

In order to evaluate how the more recent changes in composition have affected potential neurotoxicity, hydrolysates of "conventional" TCP, "purified" TCPs used in commerce but not used in jet engine oils, and newer "low-toxicity" TCPs were analyzed. Such a comparison across a wide range of potencies necessitates that toxicity measures for all samples be on the same scale. In our testing approach, the Long-Evans rat was used to evaluate "conventional" and "purified" TCPs, and the hen, which is more sensitive to inhibitors of NTE, was employed to evaluate the low-toxicity TCPs. A single scale was created by expressing potency as a percent of TOCP activity. The conversion of percent NTE inhibition to its "toxic equivalent" as percent TOCP activity is shown in Table 10 for the samples subjected to compositional analysis. TCP 86325 was expended prior to the chemical analysis but is included in Table 10 for reference; its inhibition of S-CHE and B-NTE is similar to that of 89105. At equal milligram per kilogram doses, the B-NTE inhibitory activity of "conventional" TCPs ranged from 16% to 37% of TOCP activity, the two "purified" TCPs showed 10 and 6% that of TOCP, and the low-toxicity TCPs ranged from 1.6% to 2.4% that of TOCP. The maximum amount of TOCP that could have been present in these groups of TCPs, if all of the *o*-cresyl residues found by chemical analysis (Table 11) were present as TOCP, would be 0.09 to 0.03% for the "conventional" TCPs, 0.003% to not detectable for the "purified" TCPs, and 0.03% to not detectable for the "low-toxicity" TCPs. However, most of the *o*-cresyl residues would be expected to occur in mixed isomers, mostly with a single *o*-cresyl substitution per molecule.

TABLE 10. Commercial TCP Samples, of Potential Use in Jet Engine Oil, Obtained for Testing Between 1985 and 1992

	Inhibition of brain NTE (%)		Dose of TOCP equivalent to 2 g/kg of TCP[a]	
Sample number	Rat	Hen	mg/kg	(%)[e]
A. Conventional[b]				
86325	76		680	(34)
88267	56		320	(16)
88269	60		370	(18)
88311	64		430	(22)
88356	76		680	(34)
89105	78		740	(37)
92053	56		320	(16)
B. Purified[c]				
88360	37		190	(10)
88593	27		120	(6)
C. Low-toxicity[d]				
96297 (89656)	3	25	32	(1.6)
96298		29	38	(1.9)
96299		34	46	(2.3)
97004		36	49	(2.4)
D. TOCP	93.4		2000	(100)

[a]TOCP dose equivalent (mg/kg body weight) that would produce the same percent inhibition of brain NTE as 2 g/kg/body weight of TCP. Samples A and B were evaluated in the rat and C in the hen. The hen was 3.25 times as sensitive to TOCP as the rat in our studies. These doses were derived from the dose response relationships in Figure 1.

[b]TCPs of the type used in jet engine oils for the past 15 yr.

[c]Partially purified TCPs not known to have been used in jet engine oil.

[d]High-purity TCPs, now in jet engine oil service for certain applications.

[e]Activity expressed as percent of TOCP activity.

The phenolic components of the TCPs in Table 10 are presented in Table 11 along with the percent TOCP activity. Fourteen phenolic compounds were detected in the hydrolysates. The components that might confer neurotoxicity on TCP are those with substitutions in the *ortho* position. The *ortho*-phenols and *ortho*-xylenols are summed in the two columns on the right side of Table 11. The "purified" TCPs showed reduced levels of all of the substituted phenols except *meta*- and *para*-cresol, which do not contribute to TCP neurotoxicity. Low levels of *ortho*-cresol and *ortho*-xylenols were probably responsible for the residual 6–10% TOCP activity shown in Tables 10 and 11. Even though xylenols confer some neurotoxic potential on TCP, the *ortho*-cresol moieties contribute substantially more to the activity. For both the "conventional" and "purified" TCPs, NTE inhibition was highly correlated with total *ortho*-substituted

TABLE 11. Analysis of TCP Hydrolysates

Sample description	Sample number	B-NTE inhibition as % TOCP activity	Phenolic components[a] (mean wt% + SEM)						
			Phenol	2-Me-phenol	3-Me-phenol	4-Me-phenol	2-Et-phenol	2,5-Xylenol	3-Et-phenol
A. Conventional	88267	16	0	0.27	55.72	25.71	1.40	2.99	2.88
	88269	18	0	0.10	56.82	22.48	0.60	3.24	3.63
	88356	34	0	0.17	53.76	27.98	2.07	3.00	3.12
	89105	37	0.05	0.15	56.00	28.31	2.13	2.91	3.42
	92053	16	0.19	0.08	56.12	24.27	0.10	0.54	4.14
			0.05 ± 0.04	0.16 ± 0.07	55.68 ± 1.15	25.75 ± 2.47	1.26 ± 0.90	2.54 ± 1.12	3.44 ± 0.49
B. Purified	88360	10	0	0	65.37	31.25	0.61	0.29	0.25
	88593	6.0	0.04	0.08	62.40	35.04	0.07	0.13	0
			0.02 ± 0.03	0.04 ± 0.06[d]	63.89 ± 2.10[d]	33.15 ± 2.68[d]	0.34 ± 0.38	0.21 ± 0.11[d]	0.13 ± 0.18[d]
C. Low-toxicity[e]	89656	1.6	0	0.06	61.11	36.61	0	0	0
	96297	1.6	0	0.07	62.09	37.57	0	0	0
	96298	1.9	0	0.09	68.39	31.28	0.03	0.09	0
	96299	2.3	0.47	0	55.74	43.17	0.22	0.06	0
	97004	2.4	0.12	0.06	62.49	37.00	0	0.05	0
			0.12 ± 0.20	0.06 ± 0.03	61.96 ± 4.50	37.13 ± 4.22	0.05 ± 0.10	0.04 ± 0.04	0

[a]Me = methyl, Et - ethyl, Pr = propyl. Methyl phenol = cresol.
[b]Sum of area % for 2-Me-, 2-Et-, and 2-Pr-phenol.
[c]Sum of area % for 2,5-, 2,4-, 2,6-, and 2,3-xylenol.

phenol levels (Figure 3). There was no correlation, however, between total *ortho*-xylenol content and NTE inhibition (result not shown).

Upon hydrolysis, the "low-toxicity" TCPs produced significantly lower levels of 2,4-xylenol, 3,5-xylenol, and total *ortho*-xylenols than the "purified" TCPs, but *ortho*-methyl phenol and *ortho*-ethyl phenol were not significantly different. The *meta*- and *para*-cresols together represented 99.1% of the total phenolics. It is likely that the lower toxicity of the "low-toxicity" TCPs over the "purified" TCPs results from reduced levels of *ortho*-xylenols. The very low, but consistently observed, inhibition of B-NTE seen with "low-toxicity" TCPs cannot be explained, since they are derived almost entirely from *meta*- and *para*-cresols and were expected to be completely inactive.

One sample of each of the three TCP groups was analyzed by GC-MS, and results are shown in Figure 4. TCP 86325, a "conventional" TCP, was a complex mixture of triarylphosphates of 3 predominant molecular weights: 368 (64%), 382 (32%), and 396 (4%). The tracings are displayed in panels A_1 and A_2. Molecular weight (MW) 368 corresponds to pure TCP; the higher

2,4-Xylenol	3,5-Xylenol	4-Me-phenol	2,6-Xylenol	2,3-Xylenol	3,4-Xylenol	2-Pr-phenol	Unknown	Total *ortho* phenol[b]	Total *ortho* xylenol[c]
3.94	2.78	0.28	1.54	1.18	0.18	0.05	1.08	1.72	9.65
3.78	4.67	0.09	1.98	1.50	0.15	0.07	0.89	0.77	10.5
3.40	1.14	1.37	0.45	1.10	0.10	0.05	2.29	2.29	7.95
2.66	1.50	1.44	0.29	1.04	0	0.09	0	2.38	6.90
0.75	2.03	2.41	0	1.56	5.32	0.18	2.31	0.36	2.85
2.91 ± 1.30	2.42 ± 1.40	1.12 ± 0.95	0.85 ± 0.86	1.28 ± 0.24	1.15 ± 2.33	0.09 ± 0.05	1.31 ± 0.99	1.50 ± 0.40	7.57 ± 1.34
0.35	0.17	0.06	0	0.08	0	0	1.57	0.61	0.72
0.16	0.14	0.09	0	0.06	0	0	1.79	0.15	0.29
0.26 ± 0.13[d]	0.16 ± 0.02[d]	0.08 ± 0.02[d]	0[d]	0.07 ± 0.01[d]	0	0	1.68 ± 0.16	0.38 ± 0.23[d]	0.51 ± 0.22[d]
0	0	0	0	0.20	0	0	2.02	0.06	0.2
0	0.04	0	0	0.23	0	0	0	0.07	0.23
0	0.08	0.04	0	0	0	0	0	0.12	0.09
0	0.17	0.07	0	0	0	0	0	0.22	0.06
0.09	0.01	0.05	0	0	0	0	0	0.06	0.14
0.02 ± 0.04[f]	0.06 ± 0.07[f]	0.03 ± 0.03[f]	0	0.09 ± 0.12	0	0	0.40 ± 0.9	0.11 ± 0.03	0.14 ± 0.32[f]

[d]Significantly different from A, $p < .05$.
[e]With the exception of 2-Pr-phenol, all mean values for C were significantly different from A, $p < .05$ to $p < .001$.
[f]Significantly different from B, $p < .05$.

molecular weights must include ethyl phenyl and/or xylyl groups. The "purified" TCP (88593) was a simpler mixture of 2 molecular weights, 368 (98%) and 382 (2%); these tracings are shown in panel B. In this case, the small amount of MW 382 is derived primarily from combinations of *meta*- and *para*-cresol with xylenol. These results are in good agreement with the compositional analysis of the hydrolysates shown in Table 11. Figure 4C shows the composition of the "low-toxicity" TCP (89656), in which only the *m*- and *p*-cresol derived isomers of TCP are observed.

DISCUSSION

Comparison of Neurotoxic Potential for TCPs Made Over the Past 50+ Years: Animal Studies

TCP has been used in lubricants for over 50 yr, and during this time period there have been outbreaks of OPIDN associated with ingestion of both TCP and lubricants formulated with TCP (Inoue et al., 1988). In at least one of these instances, ingestion of JEO was responsible, in Morocco
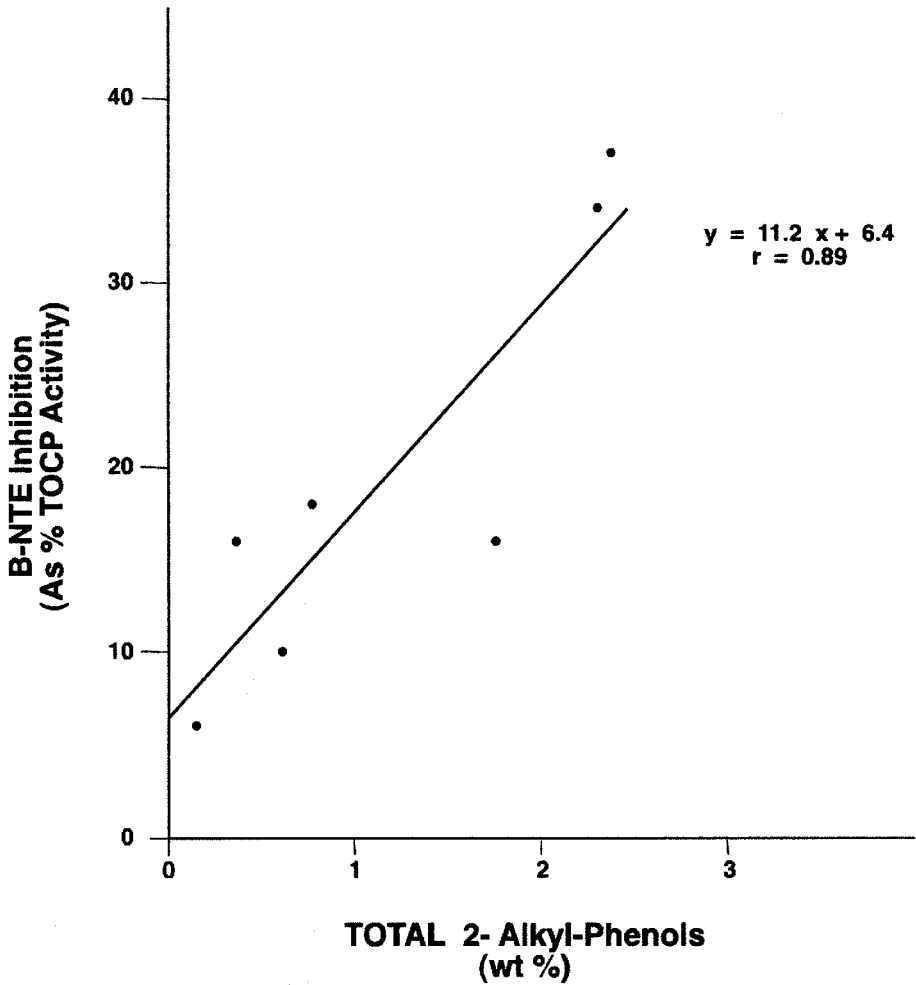
FIGURE 3. Neurotoxicity potential versus total *o*-phenol content for commercial TCPs. NTE inhibition was determined in single-dose screening studies at 2000 mg/kg in the rat.

in 1959 (Charnot & Trotemann, 1968). However, the synthesis, and therefore the composition, of the TCP mixture, has changed over time such that neurotoxic potential has been reduced. But how much less toxic is the TCP produced today than that produced years ago? By answering this question, we will be better able to assess the potential hazard and risk of JEO containing TCP. In order to do this, it is necessary to compare potential neurotoxicity of TCPs produced many years ago with those manufactured today through the use of a common scaling procedure. TCPs evaluated in hen or rat were placed on a common scale by computing TOCP equivalence, since TOCP has been tested in both species. TOCP equivalence was previously computed by Henschler and Bayer (1958) and Neumann and

Henschler (1957) for several TCPs produced in the 1940s and 1950s. TOCP equivalence can be related to potential neurotoxicity by assuming that the minimum dose, or threshold, for initiating OPIDN is equivalent to a certain percent of either B- or SC-NTE inhibition; a 70% inhibition for B-NTE and 50% for SC-NTE in the hen was found in this present study.

Thus, it is possible to obtain a TOCP equivalence for a particular TCP at 70% B-NTE inhibition from the minimal neurotoxic dose for both agents in the hen or from the potency ratios. TOCP equivalence could be similarly determined for acute, subacute and subchronic study designs. In Table 12, the acute doses required in the hen to inhibit B-NTE by 70% for 5 classes of TCPs produced at different times are presented. Classes 1–3 have been tested for OPIDN in the hen (Neumann & Henschler, 1957), and the activity versus TOCP has been expressed by these authors as "times TOCP activity." The doses projected to inhibit B-NTE by 70% were computed using the Dudek (1979) dose-response curve (sensitivity 8.35 × rat) or our own dose-response curve (sensitivity 3.25 × rat). For Classes 4 and 5, data are taken from Table 10.

The Class 1 TCP was manufactured from a crude cresol mixture (DAB 4M) containing about 30% *ortho*-cresol. This was the "torpedo oil" type responsible for numerous multiple poisonings in Germany during World War II and shortly thereafter; it was about 8–10 times more potent than TOCP itself (Henschler, 1958). The Class 2 TCPs were detected in two JEOs
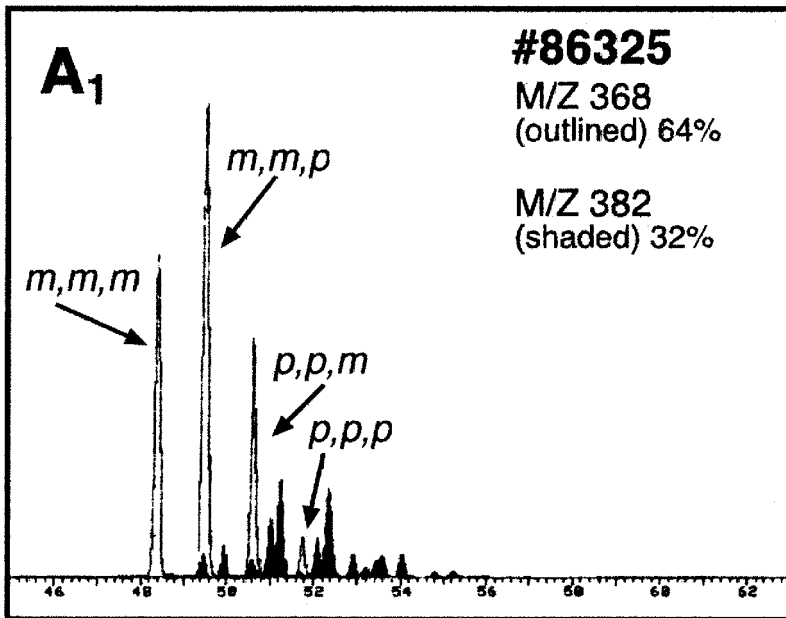

FIGURE 4. Mass spectra for three representative commercial TCPs. A_1 conventional "low-*ortho*" TCP. The retention time for TOCP in this system is 47.6 min.
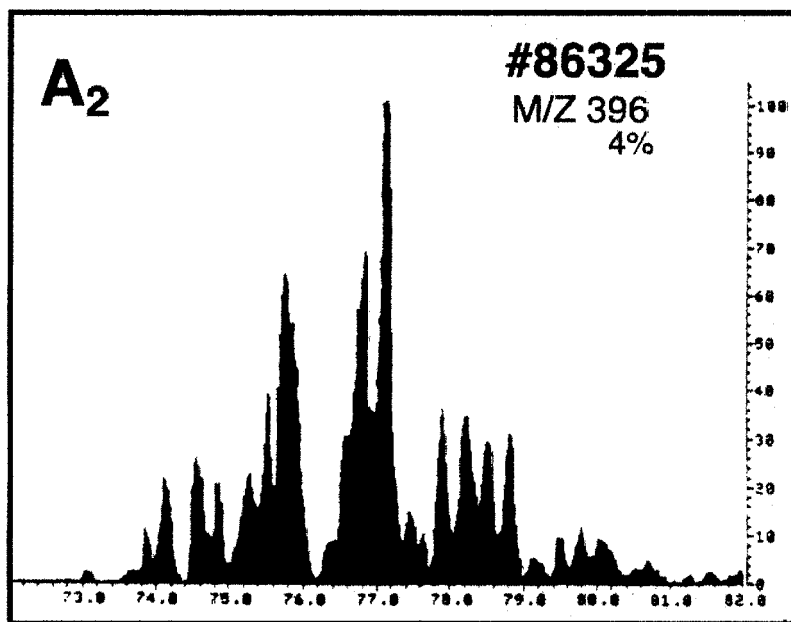
FIGURE 4. (*Continued*) Mass spectra for three representative commercial TCPs. A$_2$, conventional "low-*ortho*" TCP. The retention time for TOCP in this system is 47.6 min.

that were intentionally mixed into cooking oil and sold for food use in Morocco in 1958. Henschler and Neumann (1968) reported that toxicity of the TCPs in the Moroccan oils (B and C) were 50 and 25%, respectively, of the "torpedo oil" type TCP prepared from DAB 4M. The Class 3 TCPs were described by Henschler (1958) as modern commercial preparations of greatly reduced (~3%) *ortho*-cresol content with activity at 3% of the TCP from DAB 4M. The Class 4 "conventional" TCPs are those commonly available through 1992, and perhaps thereafter, and are represented in Table 10 by 88267 and 89105, those with the lowest and highest inhibiting activities, respectively, against B-NTE. The Class 5 "low-toxicity" TCPs are those commercially available through 1997, and perhaps thereafter, and are represented by 96297 and 97004, also of the lowest and highest inhibitory activities, respectively, against B-NTE.

The results in Table 12 show a dramatic reduction in the toxicity of TCP from that used during World War II to the low-toxicity materials available today of at least 400-fold. However, the "modern" low-*ortho*-cresol TCP described by Henschler (1958), shown in Class 3, was in the same range of activity as that for the more current "conventional" TCPs in Class 4 (and Table 10). The "low-toxicity" Class 5 TCPs are, however, at least 10- to 20-fold less potentially neurotoxic than the conventional TCPs of Class 4.

The reduction in activity from Class 1 to Class 5 is associated with changes in the phenolic mixture used for synthesizing TCP and the

introduction of processing alternatives and improved methods of purification. Effort has been focused on reducing *ortho*-cresol content in the reaction mixture and in finished TCP. Measures of *ortho*-cresol content in hydrolysates of TCP have been obtained for the five TCP classes of Table 12, and results are plotted in Figure 5. The commercial TCP from DAB 4M was synthesized from a mixture that was approximately 25% *ortho*-cresol (Henschler, 1958); the TCP of Moroccan oils A and B was from a mixture of approximately 12% *ortho*-cresol (Henschler & Neumann, 1968); and the "modern" low-*ortho* commercial preparation was from a mixture of approximately 3% *ortho*-cresol (Henschler, 1958). These reductions in *ortho*-cresol content correlated with a substantial lowering in the toxicity of the synthesized TCPs (Table 12). There was also a lower level of *ortho*-cresol content seen in hydrolysates of the "conventional" commercial production (Class 4, Table 12), but this was not associated with decreased potential toxicity. Residual toxicity of the Class 4 TCPs appears to be related largely to *ortho*-ethylphenol and perhaps to *ortho*-substituted xylenols (Table 11). Table 11 and Figure 5 show a small decrease in *ortho*-cresol between the "conventional" TCP (Class 4) and the "low-toxicity" TCP (Class 5), which is not statistically significant and is probably of too small a magnitude to impact potential neurotoxicity.

Thus, the reduction in TCP toxicity from the DAB 4M-derived material of 1940–1950 to the low-*ortho* material of 1957+ is related to reduction of
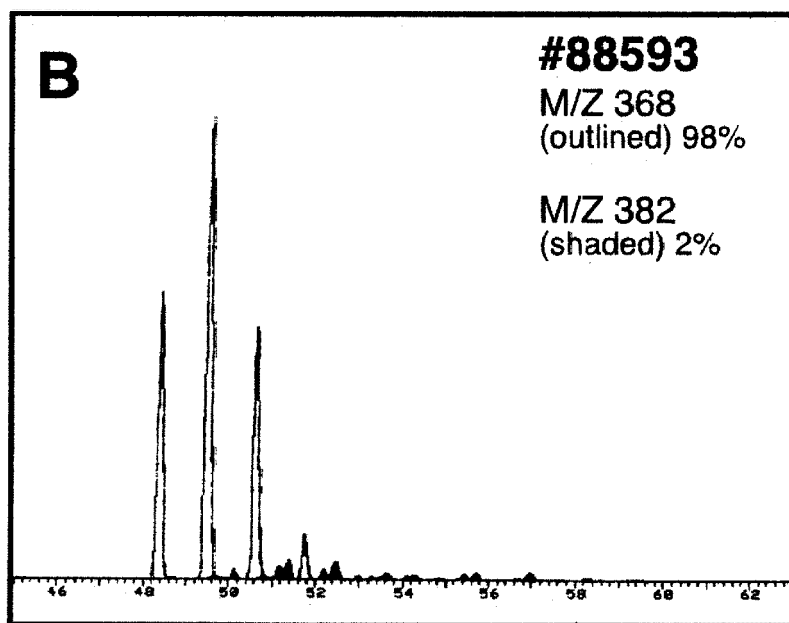

FIGURE 4. (*Continued*) Mass spectra for three representative commercial TCPs. B, "purified" TCP. The retention time for TOCP in this system is 47.6 min.
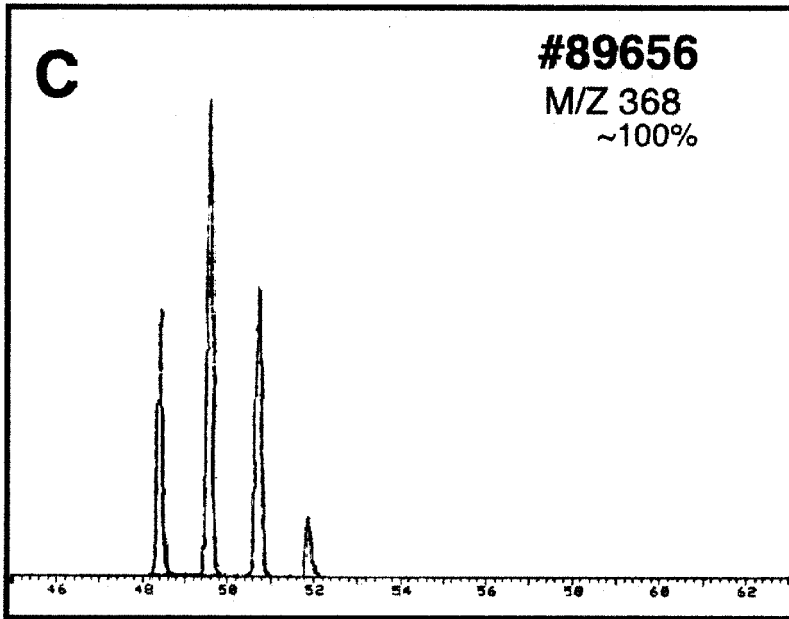
FIGURE 4. (*Continued*) Mass spectra for three representative commercial TCPs. B, "purified" TCP; C, "low-toxicity" TCP. The retention time for TOCP in this system is 47.6 min.

ortho-cresol in the reaction mixture. In contrast, hydrolysates of commercial production from 1985–1992+ showed lower levels of *ortho*-cresol content but with minimal impact on toxicity. Hydrolysates of currently manufactured "low-toxicity" TCPs have extremely low levels of all *ortho*-substituted phenols and xylenols and are composed almost entirely of *meta*- and *para*-cresols.

Comparison of Neurotoxic Potential
for TCPs Made Over the Past 50+ Years: Human Risk

Despite the large number of people who have been poisoned by TCP over more than 50 yr and the recognition that the hen is an excellent model for the syndrome of OPIDN, an acceptable quantitative relationship between hen and human with regard to induction of OPIDN has not been realized. An accurate estimate of these relative sensitivities is crucial for performing a risk assessment for TCP in humans.

A reasonable determination can be developed, as follows, from the papers by Smith et al. (1930) and Henschler (1958), which discuss the Ginger Jake outbreak of OPIDN. If it is assumed that as little as 1 oz of adulterated ginger extract containing 2.4% TCP caused OPIDN in a person (Smith et al., 1930), the single dose of TCP ingested would have been about 690 mg, or 10 mg/kg for a 70-kg person. The pharmacological and analytical investigations performed by Smith et al. (1930) ascribed all of

TABLE 12. Single Oral Dose Comparisons of Several Commercial TCPs Produced Over the Last 50–60 Years

Class	Individual samples	Approximate dates of use or manufacture	Activity expressed as times TOCP activity	Dose to inhibit NTE 70% in hen brain (mg/kg), at hen sensitivity		Dose of jet engine oil at 3% TCP to inhibit hen B-NTE by 70% dose range (g/kg)	
				8.35 × Rat[a]	3.25 × Rat[b]	Minimum	Maximum
1	Commercial TCP from DAB 4M[c]	1940–1950	8–10	8.0–6.4	20–16	0.21	0.67
2	Moroccan mass poisoning[d]	1959					
	Commercial TCP (oil B)		4–5	16–12.8	40–32	0.43	1.3
	Commercial TCP (oil C)		2–2.5	32–25.6	80–64	0.85	2.7
3	Commercial production (low *ortho*)[c]	1957+					
	TCP A		0.3–0.24	270–210	690–540	7.0	23.0
	TCP B		0.3–0.24	270–210	690–540	7.0	23.0
	TCP C		0.3–0.24	270–210	690–540	7.0	23.0
4	Commercial production (conventional)	1985–1992+					
	CRU 88267		0.16	398	1000	13.3	33.3
	CRU 89105		0.37	170	440	5.7	14.7
5	Commercial production (low toxicity)	1988–1997+					
	CRU 96297		0.016	4000	10000	133	333
	CRU 97004		0.024	2600	6700	86.7	223

[a]From Figure 1, line A.
[b]From Figure 1, line B.
[c]From Henschler (1958).
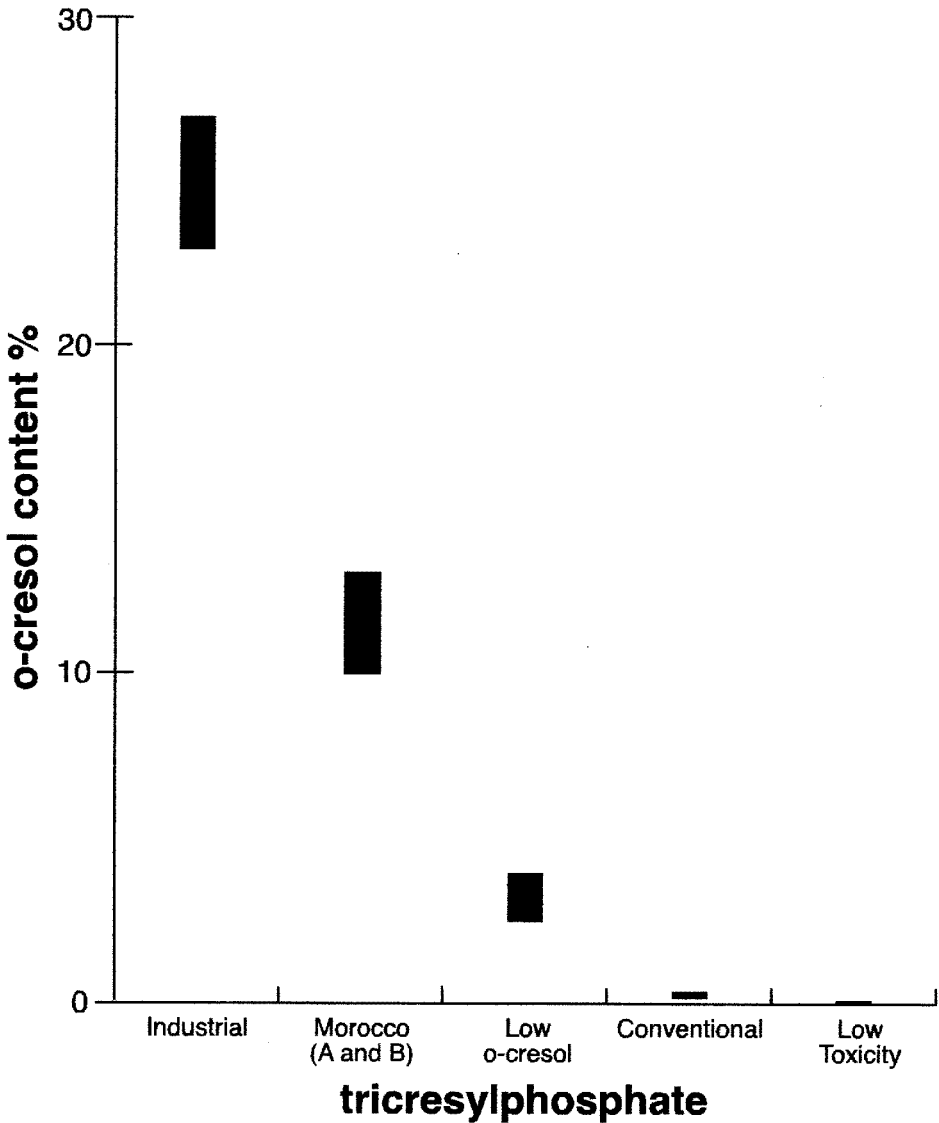[d]From Henschler and Neumann (1968).

FIGURE 5. Concentration of *o*-cresol moieties in the TCP classes of Table 12: industrial (Class 1); Morocco (Class 2); low *o*-cresol (Class 3); conventional (Class 4); and low-toxicity (Class 5).

the activity to TOCP, although it was noted that other isomers were present at low levels. A 10-mg/kg dose of TOCP is far below the dose (64 mg/kg) that produces 70% inhibition of B-NTE in the hen (Figure 1, line A). However, it is very likely that the TCP in the Ginger Jake beverage was a readily available, relatively inexpensive grade (e.g., Lyndol) with very high *ortho*-cresyl content. The spectrum of neurological symptoms of the Ginger Jake epidemic suggests a material compositionally between a

TCP of only *mono-* and di-*ortho* substitutents and that of TOCP (Henschler, 1958). This TCP was very likely to have been about 60% TOCP and 40% mixed isomers of primarily *ortho-* and *meta-*cresols. Such a TCP was responsible for poisonings in Durban, South Africa, in 1937 (Sampson, 1942) and in London, England, in 1941 (Hunter et al., 1944). A TCP mixture of this composition is about eight times as potent as TOCP in causing OPIDN in the hen (Henschler, 1958). The TOCP equivalent for this TCP, then, would be about 80 mg/kg for 70% inhibition of B-NTE (between lines A and B in Figure 1). P. H. Craig and M. L. Barth (unpublished observations) have projected a similar, but slightly higher, minimal intake to cause OPIDN in humans. These findings suggest that hen and human are about equally sensitive to both TCP and TOCP.

Although there appears to be no difference in species sensitivity between hen and human with regard to doses of TCP necessary to produce OPIDN, there may be intraspecies differences in sensitivity, and therefore it is appropriate to apply a 10-fold safety factor for intraspecies variability. It is not necessary, however, to provide an additional safety factor to protect children as a sensitive subpopulation. Men, women, and children respond to TCP poisoning; however, children (5–15 yr) show a quicker and more complete recovery from paralysis than do adults, and for adults, recovery decreases as age increases (Zinn, 1968). Thus, children appear at lower risk for irreversible neuropathology than do equally exposed adults.

By applying a 10-fold safety factor to the doses of TCP or TCP in JEO needed to inhibit B-NTE by 70%, one estimates the range of doses predicted to cause OPIDN in humans. For the purpose of risk assessment, the TCPs of concern are those of Class 4, Table 12 ("conventional" TCP) and Table 10. These "conventional" TCPs were sold for JEO use in the 1980s and 1990s with the assumption that low *ortho-*cresol content, and resulting low TOCP level, assured a very low neurotoxic potential. Some of these are still sold today for specific commercial applications. Certainly these TCPs are lower in activity than the early products, but one example (86325) produced OPIDN in the hen. There is significant batch-to-batch variability against hen and rat B-NTE (Table 10) for 1980s TCPs; 86325 was one of the most active in the rat B-NTE screen.

For Class 4 TCPs, the acute minimum toxic doses (with 10-fold safety factor) would range from 0.017 to 0.1 g/kg for neat TCP and from 0.57 to 3.3 g/kg for JEO + TCP. For a 70-kg person, the dose ranges are 1.19–7.0 g and 39.9–231 g, respectively. For TCP itself (Classes 1–4) one can visualize the ingestion of neurotoxic amounts in a heavily contaminated food product; however, for the JEO + 3% TCP, consumption would have to be 33 times greater to reach an effective toxic dose of TCP. It is very unlikely that such a large volume of JEO would be inadvertently consumed in a single dose or day.

The subchronic hen experiment with TCP 86325, a Class 4 inhibitor of B-NTE, provides the data for an evaluation of the potential risk of

OPIDN from Class 4 TCPs after multiple successive ingestions. Since B-NTE inhibition is irreversible and recovery of activity is achieved through new enzyme synthesis, repeated daily administration of a constant dose leads to cumulative inhibition, but each successive dose inhibits to a somewhat lesser degree than the one before, until a steady-state level of inhibition is reached. However, after steady state is achieved, the severity of the ataxia may progress somewhat as neurons continue to die back. Steady state for SC- and B-NTE group mean values was reached by wk 6 in our study; however, as shown in Table 7, onset and progression of ataxia varied with individual birds, as did the extent of inhibition of SC- and B-NTE (Table 8). Of groups receiving TCP, only JEO + 3% TCP showed ≥50% SC-NTE inhibition, ≥70% B-NTE inhibition, and OPIDN (Figure 3 and Tables 6 and 8). At the next lowest concentration (1% TCP), SC-NTE and B-NTE inhibition occurred but did not reach critical levels and no animals became ataxic. The projected approximate dose at the threshold for OPIDN is 2 g/kg/d of JEO +2% TCP 86325 for 10 wk.

JEO + 2% TCP was not tested, but would have been expected to produce an effect just over threshold similar to those seen with CO + 0.5% TOCP. The median time for onset of ataxia occurred after 47 d on study. This corresponds to a daily dose of 40 mg/kg TCP or of 1.34 g/kg JEO with TCP present at likely maximum use level of 3%. Including a 10-fold safety factor on dose rate, the projected minimum toxic dose of TCP 86325 and JEO + 3% TCP for producing OPIDN in a 70-kg person would be 280 mg/d and 9.4 g/d for 47 d, respectively.

There is little doubt that food products could be contaminated with levels of neat TCP such as 86325 that would pose a neurotoxic risk, but is it reasonable to suggest that a food product, such as cooking oil, contaminated with a JEO + 3% of a Class 4 TCP, such as 86325, could be consumed at rates high enough to cause OPIDN? A description of the TCP poisoning in Calcutta, India (Srivastava et al., 1990), allows us to evaluate the likelihood of a possible future poisoning from cooking oil containing JEO. In 1988, 600 people were poisoned after consuming rapeseed oil adulterated with neat TCP. On average, the affected patients consumed 94.89 g oil (which was 22–57% TCP) over 2.44 d. This equates to 8.6–22.2 g TCP/person/d. The offending TCP was not described, but was likely a Class 3 or Class 4. If one assumes a person weighs 70 kg, the dose was 123–317 mg TCP/kg/d, a level clearly within the toxic range, even for a single dose of TCP (Table 10). However, for JEO + 3% TCP and assuming a 57% contamination of rapeseed oil with JEO, daily intake per person would be 667 mg (0.67 g) TCP/d or 9.6 mg TCP/kg/d for a 70-kg person. This is higher than the 280 mg/d calculated from the results of our subchronic study (10-fold safety factor included), and therefore it was concluded that poisoning of a sensitive human population by a cooking oil contaminated with a Class 3 or a Class 4 TCP in JEO is feasible. However, there is no record of such a JEO poisoning with either Class 3

or Class 4 TCPs despite more than 40 yr of use. The massive Moroccan poisoning of 1958 was caused by a Class 2 TCP. It is important to protect against inadvertent contamination of foodstuffs and to prevent intentional diversion of obsolete or off-spec production batches, intended for disposal, to food oil use. However, it would be virtually impossible for a person to receive enough JEO + 3% Class 3 or 4 TCP in the normal workplace or in an aircraft cabin to develop OPIDN.

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