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Disposition, elimination, and metabolism of tri-*o*-cresyl phosphate following daily oral administration in Fischer 344 male rats

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Abstract. The disposition, elimination, and metabolism of 50 mg/kg (1.36 μCi/animal) of tri-*o*-cresyl [phenyl-U-^{14}C] phosphate after ten daily oral doses was investigated in adult male Fischer 344 rats. Groups of three treated animals were killed at intervals of 24, 48, 72, and 96 h after the last administrations. Generally, the highest concentrations of radioactive material were excreted via the gastrointestinal tract and the bladder, particularly at the earlier time points. Liver, adipose, epididymis, sciatic nerve tissues; plasma; and red blood cells also contained high concentrations of radioactivity. The lowest concentrations were found in brain, spleen, testes, and heart. Four days after the last dose, the rats had excreted approximately all of the cumulative dose in either urine (63.1%) or feces (36.1%). TOCP and its metabolites in urine, feces, plasma, brain, testes, kidneys, and liver were analyzed by high-performance liquid chromatography and liquid scintillation. Metabolism studies performed 24, 48, 72, and 96 h after administration of the last dose showed that TOCP was the major compound identified in brain, testes, kidneys, plasma, and liver. Liver, additionally, had high levels of di-*o*-cresyl hydrogen phosphate and *o*-cresol. TOCP and *o*-cresol were the predominant compounds in feces; only trace amounts of TOCP were detected in urine. The major metabolites in urine were di-*o*-cresyl hydrogen phosphate, *o*-cresol, and *o*-hydroxy benzoic acid. Testes in rats given ten doses had significantly more TOCP and saligenin cyclic-*o*-tolyl phosphate than those from rats given a single dose. These results may account for testicular toxicity in rats given daily oral administrations of TOCP but not following a single oral dose.

Key words: Tri-*o*-cresyl phosphate – Pharmacokinetics – Oral dosing – Rats

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Introduction

Tri-*o*-cresyl phosphate (TOCP), a compound used as a plasticizer in lacquers and varnishes, in high-speed brake lubricant as a flame retardant, and as a heat exchanger (Windholz et al. 1983), is the first documented chemical known to cause a toxic condition now referred to as organophosphorus compound-induced delayed neurotoxicity (OPIDN; Abou-Donia 1981). OPIDN was first observed in humans at the end of the 19th century, and since then an estimated 40000 human cases of delayed neurotoxicity have been attributed to TOCP (Smith et al. 1930; Abou-Donia 1981; Abou-Donia and Lapadula 1990). Some animal species e.g., chickens, cats, dogs and cows, are sensitive to OPIDN, while rodents, e.g., mice, rats, and some non-human primates are less sensitive (Abou-Donia 1981; Veronesi 1984; Lapadula et al. 1985; Somkuti et al. 1988b). Although TOCP has been in use since the 19th century, limited information regarding its absorption, distribution, and excretion in mammalian species is available. TOCP is metabolized in cats to a more potent esterase inhibitor (Taylor and Buttar 1967; Nomeir and Abou-Donia 1984, 1986b). This active metabolite, identified as saligenin cyclic-*o*-tolyl phosphate [2-(*o*-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one] (Casida et al. 1961; Eto et al. 1962; Buttar et al. 1968; Sharma and Watanabe 1974; Nomeir and Abou-Donia 1984, 1986b), has been demonstrated to be at least fivefold more toxic than TOCP to chickens (Bleiburg and Johnson 1965). TOCP has been recognized as also causing testicular toxicity in both roosters and rats (Somkuti et al. 1987a, b, c). Fischer 344 rats develop testicular lesions and decreased epididymal sperm motility and density following subchronic daily oral TOCP administration.

Since TOCP-induced testicular toxicity in the rat is produced only by daily oral administration and not by a single dose of this compound, this study was carried out to examine the role of the disposition, elimination, and metabolism of daily doses of TOCP in male Fischer 344 rats in the development of testicular toxicity.

Table 1. Concentration of radioactivity in various tissues and body fluids of male rats after ten daily oral doses of 50 mg/kg [^{14}C]TOCP[a]

Specimen	Days after end of administration							
	1		2		3		4	
	Concentration	Tissue/plasma	Concentration	Tissue/plasma	Concentration	Tissue/plasma	Concentration	Tissue/plasma
Brain	7± 1	0.2	5± 2	0.1	9± 2	0.4	6±2	0.6
Spinal cord	44± 8	1.0	9± 5	0.2	20± 6	0.9	9±3	0.9
Sciatic nerve	74± 5	1.6	10±10	0.2	38±17	1.8	12±1	1.2
Muscle	43± 6	1.0	4± 2	0.1	15± 5	0.7	16±5	1.6
Red blood cell	72± 6	1.6	36± 1	0.8	40± 6	1.9	38±5	3.8
Plasma	45± 7	1.0	43± 8	1.0	21± 3	1.0	10±3	1.0
Lung	41± 6	0.9	25± 6	0.6	30± 8	1.4	16±3	1.6
Heart	39± 8	0.9	15± 4	0.3	19± 2	0.9	24±3	2.4
Liver	70± 4	1.6	28± 1	0.7	37± 6	1.8	17±1	1.7
Kidney	68± 5	1.5	19± 3	0.4	33± 5	1.6	29±5	2.9
Urinary bladder	171±50	3.8	51± 3	1.2	26± 6	1.2	24±7	2.4
Spleen	32± 7	0.7	14± 6	0.3	23± 2	1.1	15±4	1.5
Testis	33± 5	0.7	8± 2	0.2	9± 3	0.4	19±5	1.9
Epididymis	83±14	1.8	30± 9	0.7	23± 6	1.1	14±4	1.4
Adipose/Tissue	235±62	5.2	23± 5	0.5	34± 5	1.6	29±5	2.9
Stomach	59±10	1.3	22± 2	0.5	25± 5	1.2	22±5	2.2
Small intestine	61± 8	1.4	17± 6	0.4	23± 3	1.1	12±2	1.2
Cecum	98±14	2.2	30±11	0.7	22± 3	1.0	18±2	1.8
Large intestine	71±11	1.6	15± 8	0.3	18± 2	0.8	21±6	2.1

[a] Concentrations are expressed as μg TOCP equivalent/g of fresh tissue or ml body fluid. Each value represents the mean for 6 samples from 3 animals ± SE

Materials and methods

Chemicals

TOCP, tri-*o*-cresyl-[phenyl-U-^{14}C] phosphate, with a specific activity of 6.68 mCi/mmol (radiochemical purity >98%), was synthesized by Midwest Research Institute, Kansas City, MO. Non-radioactive TOCP (99%) was obtained from Eastman Kodak Co., Rochester, NY. Other reagents were purchased from commercially available sources. The following metabolites of TOCP were prepared as described previously (Nomeir and Abou-Donia 1986 a): *o*-cresyl dihydrogen phosphate (98%), di-*o*-cresyl hydrogen phosphate (99%), saligenin cyclic-*o*-tolyl phosphate (99%), hydroxymethyl TOCP (94%), and dihydroxymethyl TOCP (92%). Salicylic acid (99%), *o*-cresol (99%), *o*-hydroxybenzaldehyde (98%), and *o*-hydroxybenzylalcohol (97%) were purchased from Aldrich Chemical Co., Milwaukee, WI.

Care and treatment of animals

Adult (10–11 weeks old) Fischer 344 male rats approximately 200 g were used (Harlan Sprague-Dawley, Indianapolis, IN). Animals were housed in temperature-controlled (21–23° C) rooms with a 12-h light cycle before and during experimental procedures. They were supplied standard NIH-07 open formula rat chow (Ralston Purina, Co., St Louis, MO) and water ad libitum. Twelve animals received 50 mg [^{14}C]TOCP/kg/d in corn oil (1 ml/kg; 1.36 μCi/animal) for a 10-day period. From this group, three rats were killed at time intervals of 24, 48, 72, or 96 h after administration of the tenth dose.

Collection of excreta and tissues

The 96-h rats were individually housed in metabolism cages from which urine and feces were collected and recorded daily. Samples were then frozen until radioactive and chemical analyses were performed. At the specified times, animals received lethal intraperitoneal 100 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL) injections. Trunk blood was collected in heparinized tubes, and centrifuged at 2000 *g*/min for 10 min to separate the red blood cells from plasma. The tissues listed in Table 1 were excised rapidly, blotted dry, weighed, placed in plastic vials, and stored at –20° C until analyzed. The contents of the gastrointestinal tract parts were separated, and each part was washed in acetone. The wash of each part was air dried and added to its original content. Estimates of tissue weights as percentages of total body weight for an adult rat obtained from the literature were as follows: muscle 30%, plasma 4%, red blood cells 6%, and adipose 7% (Bischoff et al. 1971; Ames et al. 1975).

Determination of ^{14}C radioactivity

^{14}C radioactivity was determined with a Packard Tri-Carb Model 3255 (Packard Instrument Co., Downers Grove, IL) liquid scintillation spectrometer after preparation of samples as indicated below. All data were corrected for background interference, dilution effects, quenching, and counting efficiency. Counting efficiencies were determined by correlating external standard ratios with a series of quenched standards.

Samples from tissues, body fluids, contents of gastrointestinal tract, and homogenized excreta were oxidized by combustion in a Packard tissue oxidizer Model 306 B using 10 ml of the trapping solution Carbo-Sorb and 12 ml of the scintillation cocktail Permafluor V (Packard Instrument Co., Downers Grove, IL).

Extraction of TOCP and its metabolites

Plasma. To each plasma sample, 15 ml deionized water and 15 ml 50% H_2SO_4 were added while the solution cooled in an ice bath. This mixture was extracted immediately with 5 × 50 ml ether. The ether layers from each sample were combined, dried over anhydrous $MgSO_4$, and filtered; then 100 ml acetonitrile was added to each sample extract. The solvents were evaporated under vacuum to a volume of 3–5 ml (heat and evaporation to dryness was avoided), and the residue was further concentrated by a gentle stream of nitrogen (Nomeir and Abou-Donia 1984, 1986 b).

Brain. The three brains from each time point were combined, finely chopped with a razor blade, and homogenized in 100 ml acetonitrile for

3 min, using a Polytron Ultrasonic Homogenizer (Brinkman, Westbury, NY). The liquid was decanted and separated, and the residue was further extracted three additional times with 100 ml acetonitrile for each extraction. Tissue extracts were dried over anhydrous $MgSO_4$ and then concentrated to 50 ml under vacuum. Acetonitrile extracts were partitioned three times with 25 ml hexane to remove fats from the extracts. Hexane extracts were further partitioned with acetonitrile (3×25 ml), and the acetonitrile layers were combined with the original acetonitrile extracts; the hexane layer was discarded. Acetonitrile extracts were further evaporated under vacuum to approximately 3 ml and then evaporated by a gentle stream of nitrogen to an appropriate volume prior to high-performance liquid chromatographic (HPLC) analysis.

Liver, testes, and kidneys. Each of these tissues was chopped individually with a razor blade and homogenized for 5 min in 300 ml acetonitrile in the Polytron. The homogenized samples were left overnight; then the liquid was decanted and separated, and the residue was further extracted three additional times using 300 ml acetonitrile for each extraction. The extracts were treated as described above for brain.

Urine and feces. The urine samples collected daily from each of the three 96 h animals were combined. Fecal samples were combined in a similar manner and homogenized in 4 vol ethyl acetate. Urine was extracted in 9 vol ethyl acetate. The radioactivity extracted in the ethyl acetate was designated as "non-conjugated metabolites". The residual excreta were next subjected to hydrolysis with β-glucuronidase (EC 3.2.1.31) from bovine liver type B-1 (Sigma Chemical Co.) at pH 4.5 and 45° C for 18 h, followed by ethyl acetate extraction to yield the glucuronides. Excreta were then incubated with sulfatase (EC 3.1.6.1) from limpets type V (Sigma Chemical Co.) at 37° C for 24 h and subsequently extracted with ethyl acetate to produce the sulfate fraction. Exposure to hot 2 N sulfuric acid for 30 min, followed by ethyl acetate extraction after cooling, gave a hot acid-hydrolyzed fraction. Radioactivity remaining in the aqueous fraction was considered a water-soluble fraction (Abou-Donia et al. 1983a, b).

Analysis of TOCP and its metabolites

TOCP and its metabolites were analyzed by reversed-phase HPLC and liquid scintillation counting as described previously (Nomeir and Abou-Donia 1983). Briefly, TOCP and its metabolites were separated using a C_{18} cartridge fitted into a radial compression separation system. The mobile phase was a linear gradient of 25–80% acetonitrile in 2% aqueous acetic acid in 22 min at a solvent flow rate of 1.3 ml/min. The compounds were detected by monitoring the ultraviolet absorbance of the column eluates at 254 nm. A mixture of authentic standards of TOCP and nine of its metabolites was injected in the HPLC along with the various tissue extracts. The radioactivity eluted from the HPLC was collected in scintillation vials. To each vial was added 15 ml scintillation fluid, which consisted of ethylene glycol monomethyl ether/toluene, (2:1 v/v) containing 1.67 g PPO and 67 mg POPOP/l.

Kinetic analysis

Plotting TOCP concentration (on logarithm scale) versus time (on linear scale) for analyzed tissues and plasma resulted in a straight line. The half-life of TOCP was calculated from the elimination rate constant K. This K value was obtained by linear regression of the exponential decline in TOCP concentration with time, using the expression $t_{1/2} = 0.693/K$ (Gibaldi and Perrier 1982).

Results

Clinical and necropsy observations

No signs of acute cholinergic or delayed neurotoxic effects were observed throughout the experiment. Consumption of feed and water was the same for treated and control rats. Tissues of treated animals were comparable to those of controls with respect to color, shape, and size. No microscopic examination was carried out for any tissue.

Recovery of radioactivity

The average percentage recovery of ^{14}C extracted from various tissues at various time points were brain, 35%; liver, 41.4%; kidney, 43.7%; testis, 42.1%, and plasma, 33.2%. These values represent the ratios of radioactivity extracted relative to total radioactivity determined in each tissue or plasma. In general, percentage recoveries were higher at early time points; they decreased at later points.

Tissue distribution of radioactivity

TOCP was readily absorbed and subsequently distributed throughout the body. Table 1 indicates the concentration at 24, 48, 72, and 96 h in various tissues after the administration of ten daily oral doses of 50 mg/kg [^{14}C]TOCP. The concentration of ^{14}C in most tissues was highest 24 h after the last dose and lowest 48 h after the last dose. Most tissues showed increased level of radioactivity compared to the 48 h timepoint, which decreased 24 h later. Concentrations of radioactivities were generally higher in tissues than in plasma (Table 1). Testes and nervous tissues, targets for testical and delayed neurotoxicity, respectively, exhibited the least concentration of radioactivity. Among neural tissues, the sciatic nerve contained the highest concentration of radioactivity at 24, 48, 72, and 96 h following administration. The most striking feature of the 24 h data is the high concentrations of ^{14}C in the urinary bladder and adipose tissue. Notably high levels of ^{14}C were also found in the liver, cecum, sciatic nerve, epididymis, kidney, and red blood cells (Table 1). The largest percentages of total radioactivity were found in muscle (2.9%) and adipose (3.7%) tissue (Table 2). Intermediate amounts of ^{14}C were found in all gastrointestinal tract tissues and contents. However, these tissues contained the lowest overall ^{14}C concentrations. The half-life of the total radioactivity in tissues yielded a value of 2.5 days.

Maximum ^{14}C TOCP levels in tissue generally exceeded that in plasma by 1.3- to 5.2-fold, indicating that analyzed tissues have greater affinity for TOCP than do plasma components (Table 1), except for brain, spleen, lung, heart and testis. A slightly greater affinity was seen for the epididymis (compared to plasma).

The total amount of radioactivity in analyzed tissues reached its maximum level (11.5% of the applied dose) at 24 h following administration (Table 2). The total ^{14}C remaining after 48 h was lower (3.3% of the total dose) than that observed at the earlier time point. By 96 h, the radioactivity concentration in most tissues decreased significantly. Only 1.6% of the total dose remained; the lowest total amount of radioactivity remaining in neural tissues, lungs, heart, liver, bladder, spleen, and epididymis, while the red blood cells, muscle, liver, and adipose still contained relatively high amounts.

Table 2. Distribution of radioactivity in tissues, body fluids, and gastrointestinal tract contents of male rats following ten daily oral doses of 50 mg/kg [^{14}C]TOCP[a]

Specimen	Days after end of administration							
	1	%[b]	2	%[b]	3	%[b]	4	%[b]
Brain	13± 2	0.012	10± 5	0.010	16± 5	0.016	14± 3	0.013
Spinal cord	17± 2	0.016	4± 2	0.004	7± 2	0.007	5± 2	0.005
Sciatic nerve	5± 1	0.005	1± 1	0.001	1± 1	0.001	4± 2	0.004
Muscle[c]	3059±386	2.905	1054±144	1.031	611±105	0.593	263±24	0.247
Red blood cell[c]	1028± 86	0.976	494± 19	0.483	558± 50	0.542	455±48	0.427
Plasma[c]	429± 60	0.407	404± 40	0.395	194± 16	0.189	79± 8	0.074
Lung	47± 8	0.045	27± 8	0.026	32± 8	0.310	12± 3	0.011
Heart	31± 6	0.029	11± 3	0.011	15± 2	0.015	17± 2	0.016
Liver	728± 42	0.691	290± 29	0.284	342± 36	0.332	138± 3	0.130
Kidney	152± 17	0.144	41± 8	0.040	56± 6	0.054	52± 7	0.049
Urinary bladder	105± 14	0.100	14± 1	0.014	5± 1	0.005	3± 1	0.003
Spleen	18± 4	0.017	8± 3	0.008	12± 1	0.012	7± 2	0.007
Testis	86± 16	0.082	19± 6	0.019	22± 8	0.021	47±12	0.044
Epididymis	38± 6	0.036	14± 4	0.014	9± 2	0.009	5± 1	0.005
Adipose tissue[c]	3919±510	3.720	368± 41	0.036	546± 55	0.530	380±31	0.357
Stomach	103± 10	0.980	37± 5	0.036	30± 6	0.029	28± 3	0.026
Small intestine	452± 55	0.429	104± 12	0.102	153± 15	0.149	75±18	0.070
Cecum	345± 34	0.328	78± 8	0.076	47± 7	0.046	30± 3	0.028
Large intestine	100± 10	0.095	16± 2	0.016	19± 2	0.018	26± 9	0.024
Stomach contents	175± 23	0.166	13± 2	0.013	54± 14	0.052	3± 1	0.003
Small intestine contents	535± 52	0.508	154± 16	0.151	199± 20	0.193	30± 4	0.028
Cecum contents	476± 52	0.452	134± 15	0.131	158± 29	0.153	42± 5	0.039
Large intestine contents	198± 29	0.188	54± 6	0.053	25± 8	0.024	10± 2	0.009
Total		11.454		3.278		3.021		1.619

[a] Radioactivity is expressed as μg TOCP equivalent/tissue or total body fluid or total gastrointestinal tract contents ± SE of 6 determinations from 3 rats

[b] % total administered dose

[c] These values are calculated as follows: muscle 300 g/kg, plasma 40 ml/kg, red blood cells 60 g/kg, and adipose 70 g/kg as described in the Methods

TOCP and its metabolites in tissues

TOCP and its metabolites were identified and quantified in the brain, liver, kidneys, testes, and plasma (Table 3). TOCP was detected at all time points in liver, testis, brain, and plasma. TOCP concentration in analyzed tissues and plasma declined monoexponentially as a function of time. The results indicate that TOCP was distributed according to a one-compartment open model system and was eliminated by apparent first-order kinetics. Calculated half-life values for TOCP were similar in all analyzed tissues and ranged from 0.49 to 0.98 days (Table 3). Testis, kidney, and liver contained higher amounts of TOCP and metabolites when compared to plasma and brain. TOCP appeared to be the predominant compound in kidneys and testis. The active metabolite, saligenin cyclic-*o*-tolyl phosphate, was also found in appreciable amounts, as were di-*o*-cresyl hydrogen phosphate, *o*-cresyl dihydrogen phosphate, *o*-hydroxybenzaldehyde, *o*-hydroxybenzoic acid, and hydroxymethyl TOCP. The major metabolites identified in liver were di-*o*-cresyl hydrogen phosphate and *o*-cresol; while lesser amounts of *o*-hydroxybenzyl alcohol, *o*-hydroxy benzaldehyde, *o*-hydroxybenzoic acid, and hydroxymethyl TOCP were found (Table 3). Generally, TOCP and its various metabolites decreased in a time-dependent fashion in all tissues analyzed. Brain was consistently low in all amounts of metabolites, while plasma had appreciable amounts of most metabolites. Di-hydroxy methyl TOCP was not found in most tissues.

Excretion of TOCP and radioactivity

Figure 1 presents the cumulative percentage of radioactivity excreted in the urine and feces from the three 96 h animals. Urine contained more radioactivity (63.1%) than the feces (36.1%); by the end of the experiment, almost all of the administered dose had been excreted. The radioactivity extracted from the urine and feces was analyzed by HPLC and liquid scintillation counting before and after sequential enzymatic and non-enzymatic analysis (Tables 4 and 5). Most of the radioactivity in urine was present in the hot acid hydrolysates. The predominant compound identified in the urine was di-*o*-cresyl hydrogen phosphate followed by *o*-hydroxybenzoic acid and *o*-cresol. Lesser amounts of non-conjugated metabolites were found, with sulfates and glucuronides accounting for relatively minor amounts.

In feces, non-conjugated compounds were the major form of metabolites excreted. A great amount of TOCP was also present. Substantial amounts of *o*-hydroxybenzoic acid and unknowns were found. Glucuronides accounted for minor amounts of metabolites. Sulfates and hot acid hydrolysates were also very low.

Table 3. Concentration[a] of TOCP and metabolites in tissues of male Fischer 344 rats treated orally with ten daily 50 mg/kg doses of [^{14}C]TOCP (± SE)

Tissue and days	Metabolites[b]									
	Di-*o*-cresyl hydrogen phosphate	*o*-Cresyl dihydrogen phosphate	*o*-Cresol	*o*-Hydroxy-benzyl alcohol	*o*-Hydroxy-benzaldehyde	*o*-Hydroxy-benzoic acid	Saligenin cyclic-*o*-tolyl phosphate	Hydroxy-methyl TOCP	Dihydroxy-methyl TOCP	TOCP
Brain										
1	62±7	0.0	127± 12	515±49	162± 14	282± 18	125± 13	263± 47	0.0	380± 21
2	0.0	0.0	112± 13	125±18	12± 1	121± 12	98± 7	54± 7	0.0	145± 14
3	0.0	0.0	0.0	8	0.0	73± 5	17± 1	6	0.0	26± 5
4	0.0	0.0	0.0	0.0	0.0	0.0	13	7	0.0	6
Liver										
1	342± 34	86± 8	387± 32	135±14	188± 25	228± 23	44± 3	105± 21	4	253± 35
2	187± 22	80± 9	277± 45	81±16	144± 12	197± 14	46± 3	74± 6	0.0	161± 14
3	34± 6	24± 5	52± 7	52± 9	71± 6	44± 2	50± 9	31± 3	0.0	77± 12
4	25± 3	17± 2	16± 3	19± 2	25± 2	26±	6	8± 1	0.0	30± 6
Kidney										
1	1009±116	503± 69	609± 52	297±18	952± 88	1401±223	1265±145	3046±442	0.0	4175±476
2	0.0	0.0	140± 26	33± 3	255± 37	1527±187	942± 49	474± 56	0.0	243± 37
3[c]										
4	0.0	0.0	9	0.0	0.0	156± 15	632± 29	465± 28	0.0	128± 20
Testis										
1	506± 43	1631±201	1434±117	475±43	1609±234	1881±178	1674±154	685± 84	0.0	3906±367
2	59± 12	274± 41	1020±106	545±76	933± 68	1520±310	1249±233	783± 74	0.0	989± 89
3	0.0	363± 54	0.0	151±29	242± 28	1184±106	864± 87	183± 17	0.0	839± 68
4	0.0	274± 24	0.0	0.0	39± 4	116± 27	675± 75	262± 38	0.0	30± 3
Plasma										
1	336± 44	752± 23	378± 47	744±96	391± 31	284± 16	486± 34	825± 53	162±21	1476±141
2	401± 34	134± 16	68± 5	219±21	216± 18	100± 12	118± 10	742± 49	160±23	142± 14
3	5	0.0	78± 13	254±32	49± 4	128± 13	0.0	142± 16	70± 5	103± 17
4	1	0.0	0.0	29± 5	0.0	106± 22	0.0	66± 12	34± 8	30± 6

[a] Concentration was expressed as ng TOCP or metabolite per g of fresh tissue or ml of plasma

[b] Half-life values (days) for TOCP were: liver 0.98; testis 0.49; plasma 0.58; brain 0.49. Samples were pooled before extraction and analysis. The standard error does not reflect individual variations among animals, but it addresses the variability of the analysis

[c] Sample lost due to an error

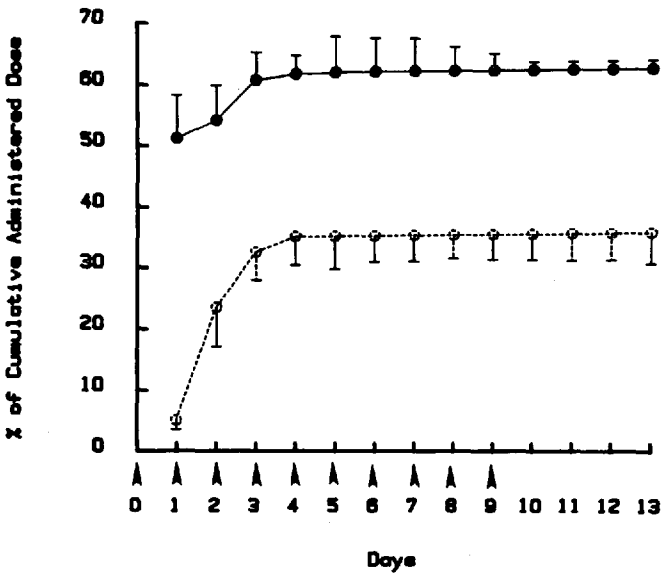

Fig. 1. Accumulated total ^{14}C in the urine and feces from rats given ten daily oral doses of 50 mg/kg [^{14}C]TOCP. *Closed circle* is urinary C^{14}, *open circle* is fecal C^{14}. Standard deviation bars are shown for each point. *Arrows* indicate the days at which [^{14}C]TOCP was administered

Discussion

This study reports the disposition, elimination, and metabolism of TOCP following ten daily 50 mg/kg oral doses of [^{14}C]TOCP in male Fischer 344 rats. Neither signs of acute cholinergic nor delayed neurotoxic effects were seen, in agreement with previous studies (Somkuti et al. 1988b). Earlier studies, however, showed that this dose regimen produced testical toxicity and male infertility in Fischer 344 rats (Somkuti et al. 1987b, c).

Daily administered oral doses of TOCP were rapidly absorbed, distributed, and eliminated. A previous study demonstrated that there was no detectable radioactivity in expired air after a single dose of 50 mg/kg [^{14}C]TOCP in Sprague Dawley rats (Nomeir et al. 1984), suggesting that complete degradation of the phenyl ring did not take place in the rat. Similar results were obtained in the cat (Nomeir and Abou-Donia 1983) and hen (Suwita and Abou-Donia 1990; Abou-Donia et al. 1990).

Lack of development of delayed neurotoxicity in these rats cannot be explained on the basis of tissue disposition or metabolism reported in this study. Although nervous tissues of these rats were among the tissues that contained low concentrations of ^{14}C 1 day after the administration of the tenth dose, they had larger concentrations of ^{14}C than

Table 4. Total μg TOCP and metabolites[a] in urine of male fischer 344 rats treated orally with ten daily 50 mg/kg doses of [^{14}C]TOCP (± SE)

Compound	Non-conjugates	Glucuronides	Sulfates	H_2SO_4 hydrosylates
Di-*o*-cresyl hydrogen phosphate	6178±524	98± 11	344±39	37920±4017
o-Cresyl dihydrogen phosphate	1283±150	45± 6	99±11	1697± 151
o-Cresol	1198±120	3173±350	804±78	11560±1052
o-Hydroxybenzyl alcohol	8321±977	270± 27	465±49	1625± 126
o-Hydroxybenzaldehyde	1706±186	245± 29	79± 8	6453± 525
o-Hydroxybenzoic acid	563± 68	4219±450	216±25	16560±1621
Saligenin cyclic-*o*-tolyl phosphate	15± 2	359± 45	24± 3	478± 33
Hydroxymethyl TOCP	66± 8	32± 3	0.0	128± 11
Dihydroxymethyl TOCP	0.0	39± 5	0.0	72± 7
TOCP	43± 5	0± 0	0.0	0± 0
Unknowns	406± 36	87± 9	0.0	892± 97
% of fraction[b]	5.0%	2.4%	0.5%	53.4%

[a] Extraction procedure described under Methods
[b] Water-soluble fraction accounted for 38.7% of total radioactivity in urine

Table 5. Total μg TOCP and metabolites[a] in feces of male Fischer 344 rats treated orally with ten daily 50 mg/kg doses of [^{14}C]TOCP (± SE)

Compound	Non-conjugates	Clucuronides	Sulfates	H_2SO_4 hydrosylates
Di-*o*-cresyl hydrogen phosphate	1586± 139	74± 9	12±2	324±33
o-Cresyl dihydrogen phosphate	4938± 521	62± 6	5±2	542±57
o-Cresol	77257±7192	388± 42	5±1	664±70
o-Hydrocybenzyl alcohol	3486± 247	177± 18	9±1	117±12
o-Hydroxybenzaldehyde	2015± 329	524± 55	10±2	75± 8
o-Hydroxybenzoic acid	15650±1571	1298±139	39±2	194±20
Saligenin cyclic-*o*-tolyl phosphate	6405± 671	376± 45	5±2	40± 5
Hydroxymethyl TOCP	4160± 430	175± 19	14±1	12± 2
Dihydroxymethyl TOCP	1538± 164	192± 20	5±1	19± 2
TOCP	57380±5801	0± 0	0±0	0± 0
Unknowns	26890±2791	1737±185	34±4	409±41
% of fraction[b]	34.8%	1.2%	0.03%	0.4%

[a] Extraction procedure described under Methods
[b] Water-soluble fraction accounted for 63.57% of total radioactivity in feces

those from rats given the same single oral dose (Nomeir et al. 1984). ^{14}C concentrations 24 h after the administration of the tenth dose were 35, 63, and 37 times those found 24 h following a single dose in the brain, spinal cord, and sciatic nerve, respectively. A considerable amount of the ^{14}C in the brain of rats given ten doses of TOCP was identified as TOCP and its active metabolite saligenin cyclic-*o*-tolyl phosphate 1 day after the last dose; only traces of these compounds were present 4 days later. The rapid disappearance of TOCP and this metabolite might contribute to the lack of delayed neurotoxicity in the rat. It is noteworthy, however, that the brain, spinal cord, and sciatic nerves from rats 1 day after the tenth dose contained 19, 64, and 30 times more radioactive material, respectively, than that found in hens 24 h after a single oral 50 mg/kg TOCP dose (Suwita et al. 1986). Also, plasma from daily treated rats contained 8 and 2 times the concentration of TOCP and its active metabolites than from the chicken 1 day after oral administration. Similar results were obtained in the cat, a species sensitive to TOCP-induced delayed neurotoxicity (Abou-Donia 1981). Brain, spinal cord, and sciatic nerves from subchronically treated rats contained 6, 44, and 21 times the concentration of ^{14}C – more than those found 1 day after a single dermal dose of [^{14}C]TOCP in the cat (Nomeir and Abou-donia 1984). Also, plasma from daily treated rats contained 0.6 and 9 times the concentration of TOCP and its active metabolite than that from the cat 1 day after dosing. These results do not explain species sensitivity to TOCP-induced delayed neurotoxicity on the basis of TOCP tissue disposition level (Abou-Donia 1979, 1980, 1983; Abou-Donia and Nomeir 1986; Whitacre et al. 1976). The present findings are consistent, however, with earlier suggestions that the low sensitivity of the rat to OPIDN may be related to differences in the delayed neurotoxicity target proteins (Abou-Donia et al. 1984; Patton et al. 1985a, b) and/or the rapid repair of the nervous tissues in the rat compared to the chicken and the cat (Veronesi 1984).

On the other hand, the testis which is vulnerable to toxicity induced by daily oral doses of TOCP but not by a single oral dose (Somkuti et al. 1987b, c), had 33 times the ^{14}C concentration in the daily-dosed animals compared to rats given a single dose. More importantly, the testis contained the highest concentration of any analyzed tissue of the metabolite saligenin cylic-*o*-tolyl phosphate. This TOCP metabolite has been recently shown to be the active agent responsible for TOCP testicular toxicity (Chabin et al. 1989). The higher concentration of this metabolite in the testis compared to plasma is consistent with the notion that it may be produced in the testis (Somkuti et al. 1988a).

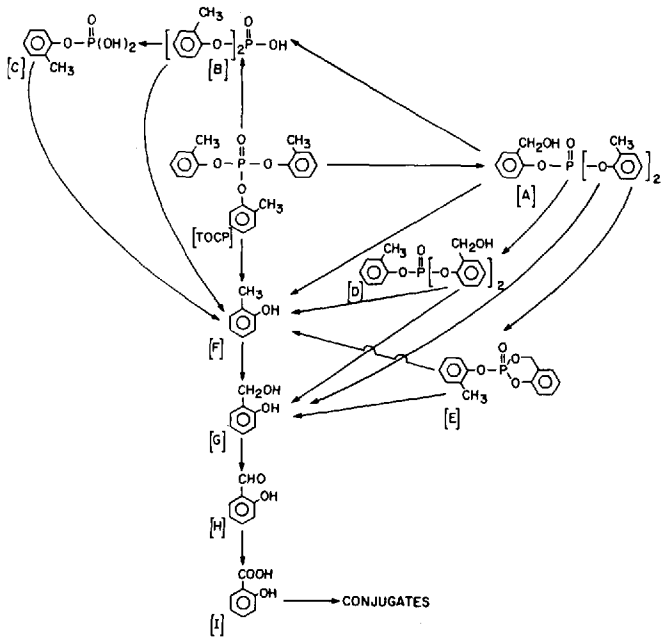

Fig. 2. Proposed metabolic pathways of TOCP in male Fischer 344 rats following subchronic oral amdinistration of 50 mg/kg [^{14}C]TOCP. *(A)* hydroxymethyl TOCP; *(B)* di-*o*-cresyl hydrogen phosphate; *(C)* *o*-cresyl dihydrogen phosphate; *(D)* dihydroxymethyl TOCP; *(E)* saligenin cyclic-*o*-tolyl phosphate; *(F)* *o-cresol; (G)* *o*-hydroxybenzyl alcohol; *(H)* *o*-hydroxybenzaldehyde; *(I)* *o*-hydroxybenzoic acid; TOCP, tri-*o*-cresyl phosphate

This suggestion is supported by the fact that testosterone synthesis involves cytochrome P-450-mediated mixed function oxidase reactions (Payne et al. 1985). It is thought that most of the non-tubular testicular cytochrome P-450 is localized in Leydig cells (Mukhtar et al. 1978). In vitro studies are presently being carried out to determine the ability of Leydig cells to metabolize TOCP.

Almost all of the administered dose of TOCP was eliminated by the end of 4 days following the administration of ten daily doses. Radioactivity, mostly as metabolites of TOCP, was excreted in the urine (63.1%) and to a lesser extent in the feces (36.1%). Most of the radioactivity recovered in the feces was present in the water-soluble fraction, followed by the nonconjugate fraction, while traces of ^{14}C were detected in the glucuronide, sulfate, and sulfuric acid hydrolysate fractions. TOCP recovered in the non-conjugated fraction may have resulted from biliary excretion or lack of absorption from the gastrointestinal tract.

On the other hand, most of the ^{14}C in the urine was present in the sulfuric acid hydrolysate followed by the water soluble fractions, while small amounts were recovered from the non-conjugate, glucuronide, or sulfate fractions. These results suggest that TOCP undergoes extensive metabolism in the rat to water soluble metabolites. This conclusion is supported by HPLC analysis, which showed that only very small amounts of TOCP were recovered in the nonconjugate fraction, while the majority of ^{14}C was associated with various TOCP metabolites.

The relatively rapid rate of excretion of TOCP-derived radioactivity is in agreement with its high rate of disappearance from plasma. Such a conclusion is also supported by the fact that the concentration level of ^{14}C in all tissues decreased substantially between 24 and 96 h after cessation of dosing. Although the liver and kidney contained comparable amounts of radioactivity, a larger amount of ^{14}C was identified as TOCP and its metabolite saligenin cyclic-*o*-tolyl phosphate in the kidney than that in the liver. The high concentration of TOCP and its metabolite in the kidney may be explained by the nonionic absorption and passive reabsorption of TOCP in the renal tubule (Milne et al. 1958), which are enhanced by its high lipid solubility (Windholz et al. 1983). The absence of renal toxicity in the presence of high concentrations of TOCP indicated that this TOCP metabolite, which has neurotoxicity and testicular toxicity, is not nephrotoxic.

On the other hand, most of the radioactivity in the liver was identified as TOCP metabolites, suggesting its prominent role in the metabolism of this compound (Hodgson and Dauterman 1980). The metabolism pattern of TOCP identified in this study suggests that it is metabolized via three pathways (Fig. 2):

1. Oxidation of one or more of the methyl groups to hydroxyl with subsequent oxidation to aldehyde carboxyl groups. This reaction is catalyzed by the microsomal mixed function oxidase system (Eto et al. 1967; Nakatsugawa and Morelli 1976).

2. Cyclization of the hydroxymethyl TOCP to form the saligenin cyclic-*o*-tolyl phosphate, which is catalyzed by plasma albumin (Eto et al. 1967).

3. Dearylation to form *o*-cresol, which may involve cytochrome P-450 similar to that reported for *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN; Lasker et al. 1982; Nomeir and Dauterman 1979).

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