Structure–Activity Relationship in the Formation of *N*-Arylacetohydroxamic Acids from Nitroso Derivatives of Chlorinated 4-Nitrodiphenyl Ether Herbicides in Boar Spermatozoa

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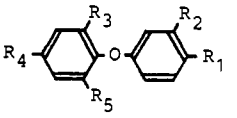
Boar spermatozoon-catalyzed formation of *N*-arylacetohydroxamic acids from nitroso derivatives of chlorinated 4-nitrodiphenyl ether herbicides and their related compounds was investigated to elucidate the structure–activity relationship. This reaction was enhanced by thiamine pyrophosphate, which was proven to be taken up by the cells from ^{31}P NMR analyses. There is no significant difference in the apparent K_m values for the nitroso compounds in the reaction. V_{max} values, however, were indicated to be affected mainly by steric hindrance of their phenoxy substituents. In the nitroso derivative of X52, an *o*-methoxy derivative of NIP, the log V_{max}/K_m was the same as that of the nitroso derivative of NIP, indicating no appreciable effect of the ortho substituent on the activity. These results indicate a toxicologically important point that the activities of the nitroso compounds in the formation of *N*-arylacetohydroxamic acids increase with a decreasing number of chlorine substituents through a dechlorinative degradation pathway in the environment. Mechanisms of the title enzymatic and acid-catalyzed nonenzymatic reactions are discussed.

INTRODUCTION

Xenobiotics including N-substituted aromatic compounds possess serious toxic potentials such as mutagenicity, carcinogenicity, and teratogenicity. NIP, a chlorinated 4-nitrodiphenyl ether herbicide (Figure 1), has been reported to cause the above-mentioned toxicities (Miyauchi et al., 1983, 1984; Milman et al., 1978; Gray et al., 1982; Hurt et al., 1983). From the viewpoints of environmental pollution and toxicology, studies on photolysis (Nakagawa and Crosby, 1974; Draper and Casida, 1985), metabolic pathways in animals (Hunt et al., 1977), and degradation in soil (Aizawa, 1982) of NIP indicate that nitro reduction, successive dechlorination, ring hydroxylation, and cleavage of ether linkage are the main degradation pathways.

As for mutagenicity of the herbicides (Miyauchi et al., 1983, 1984; Draper and Casida, 1983), it has been shown that the hydroxylamino and *N*-acetohydroxamic acid derivatives as well as the amino and nitroso derivatives are potent mutagens and that the mutagenicity increases with a decreasing number of chlorine on the phenoxy group in the molecules. These results indicate that the toxicities of the herbicides are strongly affected by both their functional groups derived from the nitro group and the number of chlorine substituents on the phenoxy group in the molecules.

N-Arylhydroxylamines and *N*-arylacetohydroxamic acids have been thought to play important roles in both cancers and mutations induced by N-substituted aromatic compounds (Frederick et al., 1985; Lotlikar, 1985; Kadlubar, 1991). As for metabolic pathways leading to the formation of proximate carcinogenic *N*-arylacetohydroxamic acids, two have been clarified: cytochrome P-450-mediated N-oxidation of acetamidoarenes (Lotlikar, 1985) and biotransformation of aromatic nitroso compounds by pyruvate decarboxylase (Corbett and Chipko, 1980). Recently, we reported the formation of *N*-arylacetohydroxamic acids from aromatic nitroso compounds and pyruvate through a pathway similar to the latter in mammalian spermatozoa (Yoshioka et al., 1989), which were known to be lacking in monooxygenase activity



				R_1	
R_2	R_3	R_4	R_5	NO_2	NO
H	H	H	H	P	P-NO
H	Cl	H	H	3Cl	3Cl-NO
H	H	Cl	H	4Cl	4Cl-NO
H	Cl	Cl	H	NIP	NIP-NO
OMe	Cl	Cl	H	X52	X52-NO
H	Cl	Cl	Cl	MO	MO-NO
H	H	F	H	4F	4F-NO
H	H	Me	H	4Me	4Me-NO

Figure 1. Structures and abbreviations of substituted 4-nitro and 4-nitrosodiphenyl ether derivatives.

(Mukhtar et al., 1978). This biotransformation seems to be mediated by pyruvate dehydrogenase complex (PDHC) in the cells, a common enzyme for aerobia including plants and animals. Considering the widespread use of the herbicides, this type of reaction may occur in the environment.

In this paper, boar spermatozoon-catalyzed formation of *N*-arylacetohydroxamic acids from a series of nitroso derivatives of the chlorinated 4-nitrodiphenyl ether herbicides and their related compounds has been investigated to evaluate the structure–activity relationship. In addition, it has been shown that the activity is enhanced by the addition of thiamin pyrophosphate (TPP), a cofactor for the enzymatic reaction (Yoshioka et al., 1989). From the nondisruptive ^{31}P NMR analyses, it has been shown that TPP is taken up by boar spermatozoa under the conditions used.

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MATERIALS AND METHODS

Apparatus. ^1H NMR and ^{31}P NMR spectra were recorded on JEOL JNM-GX270 and JNM-EX400 (operating at 162 MHz of ^{31}P frequency) spectrometers, respectively. Mass spectra were recorded on a Hitachi M-2000 spectrometer. HPLC was performed with a Shimadzu LC-5A liquid chromatograph equipped with an SPD-2A spectrophotometric detector and a column of LiChrosorb RP-8 (7 μm) (Merck; 4.0 × 250 mm).

Chemicals. Sodium pyruvate and TPP were obtained from Merck and Sigma, respectively. Nitrosobenzene was obtained from Tokyo Kasei Co. (Tokyo, Japan). Thiamin thiazolone pyrophosphate (TTPP) was prepared according to the reported procedure (Kluger et al., 1984). 4-Chloronitrosobenzene and 4-methylnitrosobenzene were synthesized as described (Lutz and Lytton, 1937). *N*-(4-Methylphenyl)acetohydroxamic acid (Mangold and Hanna, 1982) was synthesized as described. 4-(4-Methylphenoxy)nitrobenzene and 4-(4-fluorophenoxy)nitrobenzene were synthesized as described (Draper and Casida, 1983). Nitroso derivatives of the above nitro compounds and X52, namely 4F-NO, 4Me-NO, and X52-NO (see Figure 1), were synthesized according to the reported procedure (Miyauchi et al., 1984) from the corresponding *N*-acetohydroxamic acid derivatives, which were synthesized according to the procedure reported previously (Kumano et al., 1986).

N-[4-(4-Fluorophenoxy)phenyl]acetohydroxamic Acid: mp 81–83 °C (pale brown prisms from ethyl acetate–hexane); ^1H NMR (DMSO-d_6, TMS) δ 2.17 (3 H, s), 7.00 (2 H, d, J = 8.9 Hz), 7.05 (2 H, dd, J = 4.2 and 8.9 Hz), 7.22 (2 H, t, J = 8.9 Hz), 7.59 (2 H, d, J = 8.9 Hz), 10.60 (1 H, s, exchangeable with D_2O); IR ν_{max} (KBr) 3100, 2850, 1620, 1500, 1380, 1300, 1250, 1100, 850 cm^{-1}; UV (EtOH) λ_{max} 258 nm (ε 15 100); mass (EI) m/z 261 (M^+), 245, 219, 202 (base peak). Anal. Calcd for $C_{14}H_{12}NO_3F$: C, 64.36; H, 4.63; N, 5.36. Found: C, 64.26; H, 4.58; N, 5.38.

N-[4-(4-Methylphenoxy)phenyl]acetohydroxamic Acid: mp 77–78 °C (white fine platelets from ethyl acetate–hexane); ^1H NMR (DMSO-d_6, TMS) δ 2.17 (3 H, s), 2.29 (3 H, s), 6.91 (2 H, d, J = 8.2 Hz), 6.96 (2 H, d, J = 8.9 Hz), 7.19 (2 H, d, J = 8.2 Hz), 7.57 (2 H, d, J = 8.9 Hz), 10.58 (1 H, s, exchangeable with D_2O); IR ν_{max} (KBr) 3100, 2900, 1620, 1500, 1480, 1380, 1240, 1090, 830 cm^{-1}; UV (EtOH) λ_{max} 259 nm (ε 15 900); mass (EI) m/z 257 (M^+), 241 (base peak), 215, 199. Anal. Calcd for $C_{15}H_{15}NO_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 70.07; H, 5.88; N, 5.38.

4F-NO: mp 61–63 °C (blue platelets from EtOH); ^1H NMR (DMSO-d_6, TMS) δ 7.17 (2 H, d, J = 8.9 Hz), 7.27–7.39 (4 H, m), 7.99 (2 H, d, J = 8.6 Hz); IR ν_{max} (KBr) 3100, 1600, 1580, 1490, 1410, 1260, 1210, 1120, 840 cm^{-1}; UV (EtOH) λ_{max} 336 nm (ε 16 800); mass (EI) m/z 217 (M^+), 203, 101 (base peak). Anal. Calcd for $C_{12}H_8NO_2F$: C, 66.36; H, 3.71; N, 6.45. Found: C, 66.28; H, 3.74; N, 6.41.

4Me-NO: mp 41–42 °C (blue scales from EtOH); ^1H NMR (DMSO-d_6, TMS) δ 2.35 (3 H, s), 7.12 (2 H, d, J = 8.5 Hz), 7.15 (2 H, d, J = 8.5 Hz), 7.32 (2 H, d, J = 8.5 Hz), 7.97 (2 H, d, J = 8.5 Hz); IR ν_{max} (KBr) 2900, 1600, 1490, 1410, 1250, 1110, 840 cm^{-1}; UV (EtOH) λ_{max} 339 nm (ε 18 700); mass (EI) m/z 213 (M^+), 199, 101 (base peak). Anal. Calcd for $C_{13}H_{11}NO_2$: C, 73.26; H, 5.20; N, 6.57. Found: C, 73.39; H, 5.17; N, 6.58.

X52-NO: mp 103–104 °C (green prisms from benzene–hexane); ^1H NMR (DMSO-d_6, TMS) δ 4.17 (3 H, s), 6.31 (1 H, dd, J = 2.3 and 9.2 Hz), 6.41 (1 H, d, J = 9.2 Hz), 7.14 (1 H, d, J = 2.3 Hz), 7.44 (1 H, d, J = 9.0 Hz), 7.56 (1 H, dd, J = 2.0 and 9.0 Hz), 7.87 (1 H, d, J = 2.0 Hz); IR ν_{max} (KBr) 3050, 1610, 1570, 1470, 1240, 1220, 1100 cm^{-1}; UV (EtOH) λ_{max} 366 nm (ε 10 800); mass (EI) m/z 301 (M^+ + 4; $^{37}Cl_2$ containing isotope peak), 299 (M^+ + 2; ^{37}Cl containing isotope peak), 297 (M^+, base peak), 240, 204, 124. Anal. Calcd for $C_{13}H_9NO_3Cl_2$: C, 52.38; H, 3.04; N, 4.70. Found: C, 52.48; H, 2.97; N, 4.73.

Other nitroso and *N*-acetohydroxamic acid derivatives of diphenyl ethers, which were used in this experiment, were prepared as reported previously (Miyauchi et al., 1984; Kumano et al., 1986, respectively).

Kinetic Experiments. Boar spermatozoa were prepared from ejaculated boar semen, obtained from Japan Agricultural Cooperatives (Sapporo, Japan), according to the previously described procedure (Yoshioka et al., 1989). Spermatozoa were suspended at ca. 10^8 cells in 1 mL of an assay medium comprising 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH buffer (pH 6.8), 20 mM $MgCl_2$, 0.5 mM TPP, 13 mM sodium pyruvate, 0.1% (w/v) Triton X-100 as a solubilizer, and a variable concentration of nitroso derivative [added as 50 μL of a bis(2-methoxyethyl) ether solution]. Each incubation was initiated by addition of spermatozoa and carried out at 30 °C with shaking (70 strokes/min) in a 2-mL screw-cap vial equipped with a Teflon-faced seal to prevent nitroso compound volatilization (Corbett and Corbett, 1986). The reaction was stopped by heating the vial in boiling water saturated with ammonium sulfate for 15 s, and then the reaction mixture was placed on ice. One milliliter of isopropyl ether (purified by passage through a basic aluminum oxide column) was added to the reaction mixture to extract the corresponding *N*-acetohydroxamic acid derivative. The mixture was shaken for 10 min and then centrifuged briefly. The organic extract was evaporated under a slow stream of N_2, and the residue was dissolved in 250 μL of an HPLC eluant for HPLC analysis. In the case of nitrosobenzene, the organic extract was directly analyzed by HPLC as described previously (Yoshioka et al., 1989). The flow rate was 1 mL/min, and the eluants, containing 0.01% (w/v) desferal mesylate (Corbett and Chipko, 1979), were as follows: MeOH–0.01 M AcOH (2:1) (v/v) for P-NO, 4Cl-NO, 4F-NO, 4Me-NO, and X52-NO; CH_3CN–MeOH–H_2O (1:9:7) (v/v) for 3Cl-NO, NIP-NO, and MO-NO. *N*-Arylacetohydroxamic acids were monitored at 254 nm. The best-fit values of K_m and V_{max} were obtained by the method of least-squares with the Taylor expansion (Sakoda and Hiromi, 1976).

Kinetic measurements for an acid-catalyzed nonenzymatic formation of *N*-arylacetohydroxamic acids were performed at 30 °C in AcOH–H_2O (9:1) (v/v) according to the previously described procedure (Sakamoto et al., 1989). The spectral data of *N*-arylacetohydroxamic acids formed were identical with those reported in this and previous papers (Miyauchi et al., 1984; Kumano et al., 1986).

TPP Uptake Experiments. TTPP-treated boar spermatozoa were prepared as follows. The spermatozoa, which had been washed twice, were incubated at 30 °C for 13 min in the presence of 20 μM TTPP. After brief centrifugation (600*g* for 2 min at 30 °C), the spermatozoal pellet was suspended in 100 mM MOPS–NaOH (pH 6.8) and then recentrifuged to remove the extracellular TTPP. The spermatozoal pellet was resuspended in the buffer and incubated at 30 °C for 15 min. After centrifugation, the pellet was finally resuspended in the buffer. TTPP-untreated control cells were also prepared by the same procedure without TTPP. At different concentrations of TPP, *N*-phenylacetohydroxamic acid formation activities of the TTPP-treated and -untreated boar spermatozoa were measured at 30 °C in 100 mM MOPS-NaOH (pH 6.8) containing 10 mM nitrosobenzene, 20 mM $MgCl_2$, and 13 mM pyruvate.

^{31}P NMR Experiments. Boar spermatozoa were sedimented by centrifugation (200*g* for 15 min), followed by washing four times with 100 mM MOPS–NaOH buffer (pH 7.4) containing 5 mM glucose and 60 mM NaCl at ambient temperature. The final washed pellet of spermatozoa (2–3 × 10^9 cells/mL) was transferred to a 10 mm o.d. NMR tube, and ^{31}P NMR analysis was performed in the presence of 2 mM TPP and 150 mM MOPS–NaOH buffer (pH 7.4) at 25 °C. A capillary containing hexamethylphosphoramide (δ = 30.17) was used as an external standard. Accumulation of data was initiated within 10 min after the addition of TPP, and six successive blocks of 900 transients (each representing 30 min of accumulation) were collected. Each spectrum was recorded with a 45° pulse angle and a repetition time of 2 s under a broad-band proton decoupling condition. The data are shown as difference spectra from the spectrum obtained in the first 30 min of accumulation.

RESULTS

Kinetics of Enzymatic Formation of *N*-Arylacetohydroxamic Acids. Boar spermatozoon-catalyzed formation of *N*-arylacetohydroxamic acids from nitroso derivatives of chlorinated 4-nitrodiphenyl ethers and their related compounds was observed in the presence of pyruvate. A typical profile of the formation of the *N*-acetohydroxamic acid derivative from P-NO is shown

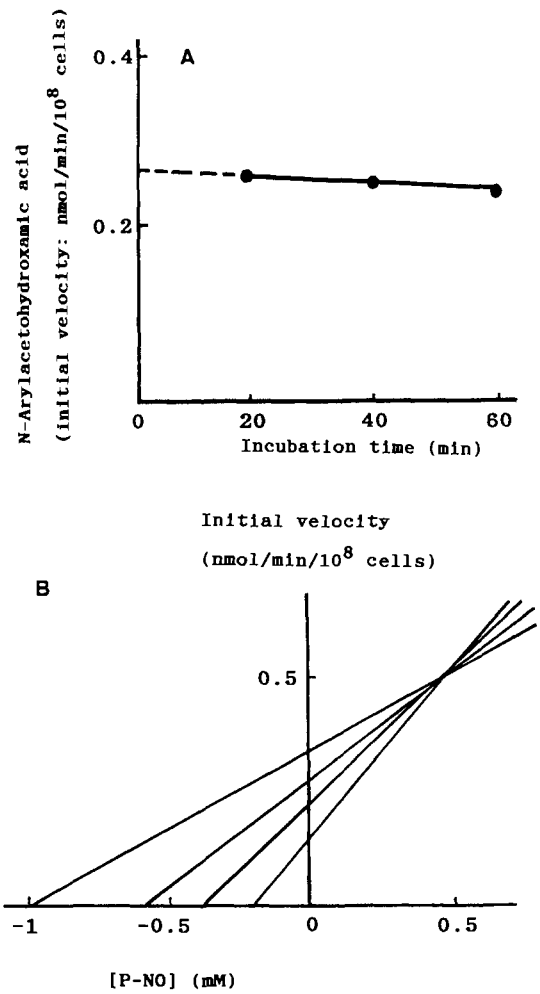

Figure 2. (A) Initial velocity of the formation of *N*-acetohydroxamic acid derivative from P-NO (0.6 mM) and pyruvate (13 mM). (B) Cornish-Bowden plot of the formation of *N*-acetohydroxamic acid derivative from P-NO in the presence of 13 mM pyruvate.

in Figure 2A. To elucidate the effect of the substituted phenoxy groups on the enzymatic reaction, kinetic parameters for the nitroso derivatives were determined by means of a Cornish-Bowden plot (Eisenthal and Cornish-Bowden, 1974) in the presence of 13 mM pyruvate, a concentration high enough to saturate the enzymatic system (Yoshioka et al., 1989). In Figure 2B, the Cornish-Bowden plot of the formation of *N*-acetohydroxamic acid derivative from P-NO is shown.

As shown in Table I, there is no significant difference in the apparent K_m values. V_{max} values, however, are affected by substituents (chloro, fluoro, and methyl) on the phenoxy group in the molecules. The order of V_{max}/K_m values, parameters indicating the catalytic efficiency of the formation of *N*-arylacetohydroxamic acids, was as follows: P-NO > 4F-NO > 4Cl-NO > 4Me-NO > 3Cl-NO > NIP-NO ≒ X52-NO > MO-NO. As for the chlorine substituent, the order indicates that the activity decreased with increasing number of chlorine on the phenoxy group in the molecules. The values of log (V_{max}/K_m) showed a good positive correlation ($r = 0.986$; $n = 7$, $P < 0.001$) with the pK_a values of phenols corresponding to the phenoxy substituent (Figure 3). The correlation equation (eq 1), in which the poorly fitted methyl-substituted derivative is omitted, is

$$\log (V_{max}/K_m) = (0.43 \pm 0.03)pK_a - (4.44 \pm 0.29) \quad (1)$$

There are two important findings. One is that the V_{max}/K_m of X52-NO, an *o*-methoxy-substituted derivative of NIP-NO, is the same as that of NIP-NO. This indicates that the *o*-methoxy substituent does not affect the activity in the boar spermatozoon-catalyzed formation of *N*-arylacetohydroxamic acid. The other is that 4Me-NO deviates from the other nitroso compounds in V_{max}/K_m values as the nitroso compound shows a lower V_{max}/K_m value than expected from the pK_a value.

Second-order rate constants, k_c, for the acid-catalyzed nonenzymatic formation of *N*-arylacetohydroxamic acids from pyruvic acid and nitroso compounds (see Table I) are also amenable to this sort of correlation analysis. It has been reported that this reaction proceeds via a nucleophilic attack by the nitrogen of nitroso compounds on α-oxo acids (Corbett and Corbett, 1980; Sakamoto et al., 1989) and that k_c values are dependent on the electronic effects (σ^+) of ring substituents (Sakamoto et al., 1989). Figure 4 shows that the k_c values of the nitrosodiphenyl ether compounds, except for X52-NO, correlate reasonably well with the pK_a listed in Table I. The correlation equation (eq 2; $r = 0.980$; $n = 7$, $P < 0.001$) is

$$\log k_c = (0.15 \pm 0.01)pK_a - (3.85 \pm 0.13) \quad (2)$$

The poor correlation of X52-NO, having an *o*-methoxy substituent, is explained by the ortho effect which reduces the nucleophilicity of the nitrogen atom of the nitroso group (Sakamoto et al., 1989). Although the pK_a values are thought to correspond to the electronic properties of the phenoxy substituents, their σ^+ constants are predicted from a linear relationship (eq 3; $r = 0.991$; $n = 4$, $P < 0.01$)

$$\log k_c = -(1.86 \pm 0.18)\sigma^+ - (3.20 \pm 0.05) \quad (3)$$

obtained by the Hammett plot of k_c of P-NO (see Table I), nitrosobenzene [$k_c = (0.77 \pm 0.03) \times 10^{-3}$ M^{-1} s^{-1}], 4-chloronitrosobenzene [$k_c = (0.33 \pm 0.01) \times 10^{-3}$ M^{-1} s^{-1}], and 4-methylnitrosobenzene [$k_c = (2.45 \pm 0.10) \times 10^{-3}$ M^{-1} s^{-1}] with their σ^+ values. In eq 3, the magnitude of the ρ value is identical with that reported previously (Sakamoto et al., 1989). These estimated σ^+ constants, shown in Table II, are reasonable and indicate that these phenoxy groups are electron-donating, with that of the 4-methylphenoxy group being the highest, and that the electron-donating ability decreases with an increase in the number of chlorine substituents. Therefore, the resulting poor correlation of 4Me-NO, shown in Figure 3, suggests that additional factor(s) other than the electronic factor should be considered in the boar spermatozoon-catalyzed reaction.

^{31}P NMR Experiment. In boar spermatozoon-catalyzed *N*-arylacetohydroxamic acid formation, it had been shown that the activity is enhanced by the addition of TPP, one of the coenzymes of PDHC which is thought to be the enzyme responsible for the reaction (Yoshioka et al., 1989). This result and the report of TPP uptake into isolated rat liver mitochondria (Barile et al., 1990) indicate that TPP is most likely taken up by boar spermatozoa as well.

When boar spermatozoa were treated with 20 μM TTPP, a specific inhibitor of TPP-dependent enzymes (Kluger et al., 1984), *N*-phenylacetohydroxamic acid formation activity of the cells was reduced to ca. 30% of the full activity. This indicates that TTPP penetrates into the cells. TTPP-untreated cells exhibited considerable activity without the addition of TPP as well as the tendency to increase in activity by the addition of TPP. In TTPP-treated cells at different concentrations of TPP, a dose-dependent recovery of the activity was more clearly

Table I. Kinetic Constants for Substituted 4-Nitrosodiphenyl Ethers in the Formation of N-Arylacetohydroxamic Acid Derivatives

nitroso compound	K_m, mM	V_{max}, nmol min^{-1} (10^8 cells)$^{-1}$	V_{max}/K_m	pK_a[a]	k_c, 10^{-3} M^{-1} s^{-1}
P-NO	0.50 ± 0.16	0.51 ± 0.06	1.00	10.00	4.85 ± 0.09
4F-NO	0.42 ± 0.02	0.27 ± 0.01	0.64	9.92	4.43 ± 0.00
4Cl-NO	0.34 ± 0.03	0.12 ± 0.01	0.36	9.38	3.75 ± 0.07
4Me-NO	0.40 ± 0.01	0.09 ± 0.01	0.23	10.26	6.17 ± 0.03
3Cl-NO	0.63 ± 0.12	0.10 ± 0.02	0.16	8.48	2.79 ± 0.05
NIP-NO	0.43 ± 0.18	0.05 ± 0.01	0.11	7.89	2.57 ± 0.07
X52-NO	0.46 ± 0.19	0.05 ± 0.01	0.11	7.89	1.33 ± 0.09
MO-NO	0.45 ± 0.11	0.02 ± 0.01	0.05	7.42	1.89 ± 0.06

[a] pK_a values of phenols corresponding to the phenoxy substituents are from Meites (1963) and Buckingham (1982).

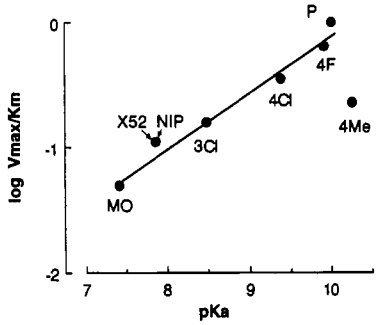

Figure 3. Plot of log (V_{max}/K_m) vs pK_a of phenols corresponding to the substituted phenoxy groups. Abbreviations in the figure indicate the nitroso derivatives of substituted diphenyl ethers.

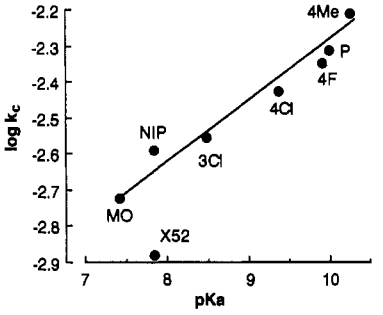

Figure 4. Plot of log k_c vs pK_a of phenols corresponding to the substituted phenoxy groups. Abbreviations in the figure indicate the nitroso derivatives of substituted diphenyl ethers.

Table II. Estimated σ^+ Values of Substituted Phenoxy Groups

substituent	σ^+	substituent	σ^+
4-Cl	−0.43	2-Cl	−0.34
4-F	−0.45	2,4-di-Cl	−0.36
4-Me	−0.53	2,4,6-tri-Cl	−0.27

observed (Figure 5). These results establish the possibility, as suspected in previous experiments (Yoshioka et al., 1989), that TPP, as well as TTPP, is taken up by boar spermatozoa. To further confirm the spermatozoal transport of TPP in vivo, nondisruptive ^{31}P NMR analyses were carried out. As shown in Figure 6A, washed boar spermatozoa exhibited three peaks corresponding to glycerylphosphocholine, inorganic phosphate, and phosphomonoesters, as reported by Robitaille et al. (1987). After the addition of TPP to the washed boar spermatozoa, two peaks (δ −6.49 and −10.62) derived from the added external TPP were observed. Since the chemical shift of the terminal phosphate of TPP is reported to be dependent on pH (Lachmann et al., 1984) and the intracellular pH of boar spermatozoa was estimated to be 7.0 ± 0.2 (Robitaille et al., 1987), intracellular TPP taken up by the cells would be observed at a higher field. As shown in Figure 6B–F, the difference spectra showed a time-dependent increase in the resonance at −6.76 ppm (peak a), a reasonable chemical shift for the terminal phosphate

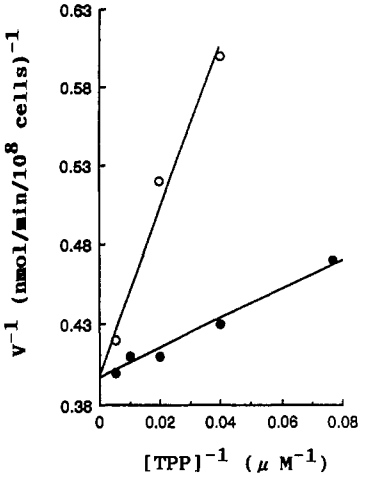

Figure 5. Effect of TPP and TTPP on N-phenylacetohydroxamic acid formation. (○) TTPP-treated boar spermatozoa; (●) TTPP-untreated (control) spermatozoa.

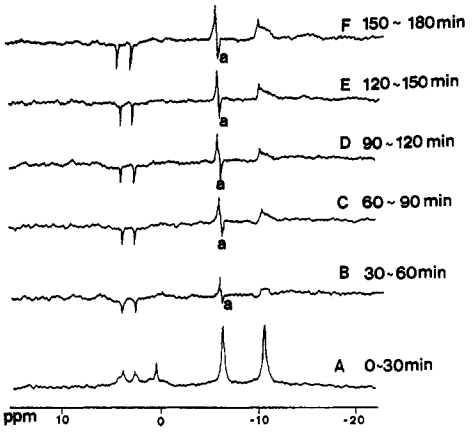

Figure 6. Difference spectra of ^{31}P NMR of boar spermatozoa in the presence of TPP.

of the intracellular TPP at pH 7.0. This result indicates that TPP is taken up by boar spermatozoa.

DISCUSSION

Toxic effects of xenobiotics, including drugs and environmental chemicals, on the reproductive system and spermatogenesis have been a focus of intensive research (Wyrobek, 1983; Schrag and Dixon, 1985; Drife, 1987; Juchau, 1989). A number of investigations on the biotransformation activity of the testis have been reported (Georgellis et al., 1989; DiBiasio et al., 1991).

One possible pathway for the formation of genotoxic metabolites of N-substituted aromatic compounds is the formation of N-arylhydroxylamines and N-arylacetohydroxamic acids (Frederick et al., 1985; Lotlikar, 1985; Kadlubar, 1991). These compounds are further activated to highly reactive electrophilic species, which are thought

Table III. Estimated E_s Values of Phenoxy and Substituted Phenoxy Groups

substituent	E_s	substituent	E_s
H	−0.85	2-Cl	−3.51
4-F	−1.52	2,4-di-Cl	−4.03
4-Cl	−2.36	2,4,6-tri-Cl	−5.18
4-Me	−2.73		

to be responsible for the genotoxic effects. In studies of the metabolism of N-substituted aromatic compounds in mammalian spermatozoa, aimed at investigating the metabolic pathway and the genotoxic effects of metabolites on the cells, a nonoxidative formation of *N*-arylacetohydroxamic acids from nitroso compounds and pyruvate has been observed (Yoshioka et al., 1989). From detailed studies with boar spermatozoa, it has been strongly suggested that the reaction is mediated by PDHC, a mitochondrial enzyme. The fact that the activity is enhanced by the addition of TPP is evidence indicating that the enzyme participates in the reaction. Although TPP has recently been shown to be taken up by isolated rat liver mitochondria (Barile et al., 1990), further studies are needed to confirm the spermatozoal transport of TPP in vivo. ^{31}P NMR analyses reported in this paper (Figure 6) suggest that TPP transports into boar spermatozoa under the conditions used.

Concerning the structure–activity relationship in boar spermatozoon-catalyzed formation of *N*-arylacetohydroxamic acid, there were large differences in the activity of the nitroso derivatives of chlorinated 4-nitrodiphenyl ethers and their related compounds (Table I). The tendency exhibited by the chlorinated 4-nitrosodiphenyl ethers to become more effective substrates with the decrease in the number of chlorine substituents has also been found in the mutagenic activities (Miyauchi et al., 1983; Yoshioka et al., 1986), the activities of methemoglobin formation (Miyauchi et al., 1981), and the transacetylation activities (Kumano et al., 1986). These activities are thought to be complicated by the electronic, lipophilic, and steric effects of the chlorine-substituted phenoxy groups in the molecules.

In the formation of *N*-arylacetohydroxamic acids, the lipophilicity of the nitroso compounds does not appear to be of significance concerning the binding and catalytic conversion of the substrate as there is no difference in the apparent K_m values (Table I). These nitroso compounds are thought to be sufficiently lipophilic to bind to an active site of PDHC, presumably the TPP binding site which is considered to be hydrophobic in nature (Wittorf and Gubler, 1970).

In the formation of *N*-arylacetohydroxamic acid catalyzed by porcine heart PDHC, effects of ring substituents of nitrosobenzenes on the V_{max}/K_m have been evaluated by the application of the Hammett (σ^+)–Taft (E_s) equation (Yoshioka and Uematsu, 1992). Since the activity of the boar spermatozoa in the formation of *N*-arylacetohydroxamic acids is thought to be mediated by PDHC, the following equation is introduced for boar spermatozoon-catalyzed reaction.

$$\log (V_{max}/K_m) = 0.33E_s + 0.56\sigma^+ + 0.56 \quad (4)$$

To estimate the steric effects of substituted phenoxy groups, the E_s constants of the phenoxy groups are calculated from eq 4. Table III shows the values. These negative estimated E_s values indicate that the greater the number of chlorines in the phenoxy group, the bulkier the corresponding phenoxy group is. To confirm the reliability of these estimated σ^+ and E_s values, k_c values listed in

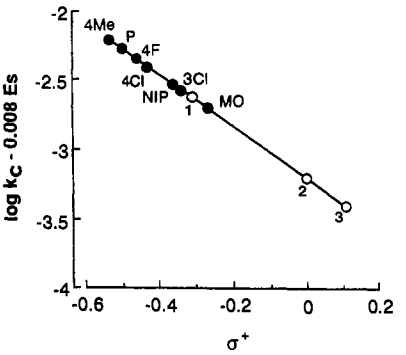

Figure 7. Correlation of log k_c vs σ^+ constants. Abbreviations in the figure indicate the nitroso derivatives of substituted diphenyl ethers. Open circles (1, 2, and 3) indicate 4-methylnitrosobenzene, nitrosobenzene, and 4-chloronitrosobenzene, respectively.

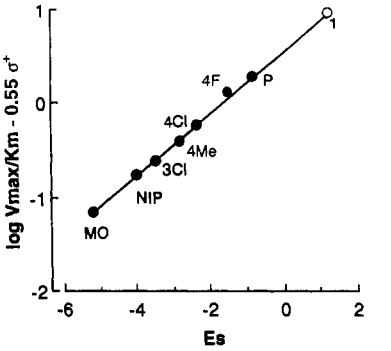

Figure 8. Correlation of log (V_{max}/K_m) vs E_s constants. Abbreviations in the figure indicate the nitroso derivatives of substituted diphenyl ethers. Open circle (1) indicates nitrosobenzene.

Table I were adopted to correlation analysis by the Hammett–Taft equation (Figure 7). As shown in eq 5 (r = 0.993; n = 10), identical ρ values can be calculated: ρ = −1.89 in eq 5 and ρ = −1.86 in eq 3.

$$\log k_c = \underset{(1.02)}{-1.89\sigma^+} + \underset{(0.05)}{0.008E_s} - 3.20 \quad (5)$$

This indicates that the estimated values are reasonable. The electronic effect was found to be important from the standard partial regression coefficients, shown in parentheses in the above equation. This is also indicated by the fact that the correlation of log k_c with the E_s parameter alone is very poor or nonexistent (r = 0.447). As shown in Figure 8 and eq 6 (r = 0.999; n = 8), the good correlation of log (V_{max}/K_m) and E_s values is obtained.

$$\log (V_{max}/K_m) = \underset{(0.95)}{0.33E_s} + \underset{(0.13)}{0.55\sigma^+} + 0.56 \quad (6)$$

From the standard partial regression coefficients, the steric effect was found to be more important than the electronic effect in the boar spermatozoon-catalyzed formation of *N*-arylacetohydroxamic acids. Low reactivities of 4-nitrosobiphenyl and 2-nitrosofluorene observed in the boar spermatozoon-catalyzed reaction (Yoshioka et al., 1989) are also explained by the steric effect of their substituents.

The proposed mechanisms for the enzymatic (Yoshioka and Uematsu, 1992) and nonenzymatic (Sakamoto et al., 1989) reactions of nitroso compounds are quite different. The former is thought to be a nucleophilic addition reaction of 2-(1-hydroxyethylidene)-TPP, an active intermediate produced in PDHC-catalyzed reaction (Kluger, 1987), toward nitroso compounds (i.e., electrophiles). On the contrary, nitroso compounds act as nucleophiles in the

latter reaction. Since substituted phenoxy groups tested are electron-donating, the nonenzymatic reactivities of all of the nitrosodiphenyl ethers tested are higher than that of nitrosobenzene. Electron-donating substituents located para to the nitroso group of nitrosobenzenes have been shown to lower the oxidation–reduction potentials (Lutz and Lytton, 1937; Kovacic et al., 1990). The fact that the enzymatic reactivities of all of the nitrosodiphenyl ethers tested are lower than that of nitrosobenzene [K_m = 0.27 ± 0.01 mM; V_{max} = 2.5 ± 0.4 nmol min^{-1} (10^8 cells)$^{-1}$; Yoshioka et al., 1989] is thought to be mainly due to the steric effect (E_s) and partly due to their lower oxidation–reduction potentials. On the other hand, ortho-substituted (both electron-donating and -withdrawing groups) nitrosobenzenes are reported to possess higher oxidation–reduction potentials than that of nitrosobenzene (Lutz and Lytton, 1937). Therefore, the unusual observation that the *o*-methoxy substituent does not affect the enzymatic activity might be explained by thinking that the steric effect of the *o*-methoxy substituent is offset by a rise in the oxidation–reduction potential.

There are many opportunities for the chlorinated 4-nitrodiphenyl ether herbicides to be converted to the nitroso derivatives in the environment: photochemical reduction (Draper and Casida, 1983); reductive metabolic pathways in intestinal microorganism (Miyauchi et al., 1980), soil (Aizawa, 1982), and both animals and plants (Hunt et al., 1977; Miyauchi et al., 1983; Shimotori and Kuwatsuka et al., 1978). Considering that the chlorinated 4-nitrodiphenyl ethers are extensively used as herbicides and that the title reaction is mediated by PDHC, the type of reaction reported here may cause important toxic effects from the following points.

(1) The formation of *N*-arylacetohydroxamic acids from nitroso compounds is thought to occur in all cells possessing mitochondria. Furthermore, there is a possibility that these compounds are proximate carcinogens of the herbicides. Genotoxic potentials of these compounds on aerobia including human beings in the environment, therefore, are of considerable importance from the viewpoint of human and environmental health.

(2) Since PDHC is a mitochondrial enzyme, this reaction may be most closely linked to toxic damages of mitochondrial function as reported in acetaminophen hepatotoxicity (Burcham and Harman, 1991). It has been shown that genotoxic agents derived from drugs and xenobiotics induce various disorders of mitochondrial functions which are associated with structural as well as functional abnormalities of mitochondria (Ebringer, 1990).

(3) The title reaction may exert toxic effects on cells by inhibiting the normal function of PDHC, a key enzyme playing central roles particularly in regard to cellular energetic metabolism, causing severe impairment to the energy metabolism of the cells. In plant cells, the disruption of the energetic metabolism of the cells might be in part concerned with herbicidal activity of these light-requiring chlorinated 4-nitrodiphenyl ethers, though this hypothetical mechanism is most likely not the primary action of these herbicides (Scalla and Matringe, 1990).

(4) Although there is no evidence for *N*-phenylacetohydroxamic acid to be further biotransformed into reactive species in boar spermatozoa under the conditions used (Yoshioka et al., 1989), the genotoxic potentials of *N*-arylacetohydroxamic acids might increase the frequency of germ cell mutation, especially in the early stage of spermatogenesis, and may cause further abnormalities in subsequent generations.

(5) From the studies on the structure–activity relationship, the finding that successively dechlorinated compounds produced in the environment are also more effective substrates for the formation of *N*-arylacetohydroxamic acid derivatives is noteworthy from a toxicological point of view.

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