Acta Medica Okayama

Volume 42, *Issue* 1 1988 *Article* 2

FEBRUARY 1988

Effect of phenol and halogenated phenols on energy transfer reactions of rat liver mitochondria.

Fumio Izushi* Takaaki Mori†

Masana Ogata‡

*Okayama University,

†Okayama University,

‡Okayama University,

Effect of phenol and halogenated phenols on energy transfer reactions of rat liver mitochondria.*

Fumio Izushi, Takaaki Mori, and Masana Ogata

Abstract

The in vitro effects of phenol and p-halogenated phenols on mitochondrial energy transfer reactions were examined using isolated rat liver mitochondria. The relationship between physiochemical properties of phenolic compounds and their effects on mitochondria were studied. Phenol and p-halogenated phenols induced the release of K+ ions from mitochondria, suggesting a change in permeability to K+ ions. A decrease in the respiratory control index, an increase in K+ release and stimulation of latent ATPase activity were observed with these compounds in the descending order of p-iodophenol, p-bromophenol, p-chlorophenol, p-fluorophenol and phenol. The concentrations of the phenolic compounds resulting in fifty percent inhibition of the respiratory control index and those resulting in fifty percent release of K+ ions significantly correlated with Hammett's substituent constant (sigma) and the hydrophobic binding constant (pi) of the compounds.

KEYWORDS: phenol, mitochondria, oxidative phosphorylation, Hammett's substituent constant, hydrophobic bindung constant

---

*PMID: 2966543 [PubMed - indexed for MEDLINE]

Acta Med Okayama 42 (1) 7-14 (1988)

Effect of Phenol and Halogenated Phenols on Energy Transfer Reactions of Rat Liver Mitochondria

Fumio Izushi*, Takaaki Mori and Masana Ogata

Department of Public Health, Okayama University Medical School, Okayama 700, Japan

The *in vitro* effects of phenol and *p*-halogenated phenols on mitochondrial energy transfer reactions were examined using isolated rat liver mitochondria. The relationship between physicochemical properties of phenolic compounds and their effects on mitochondria were studied. Phenol and *p*-halogenated phenols induced the release of K^+ ions from mitochondria, suggesting a change in permeability to K^+ ions. A decrease in the respiratory control index, an increase in K^+ release and stimulation of latent ATPase activity were observed with these compounds in the descending order of *p*-iodophenol, *p*-bromophenol, *p*-chlorophenol, *p*-fluorophenol and phenol. The concentrations of the phenolic compounds resulting in fifty percent inhibition of the respiratory control index and those resulting in fifty percent release of K^+ ions significantly correlated with Hammett's substituent constant (σ) and the hydrophobic binding constant (π) of the compounds.

Key words : phenol, mitochondria, oxidative phosphorylation, Hammett's substituent constant, hydrophobic binding constant

Phenol is widely used for the production of drugs, pharmaceutical and agricultural preparations, synthetic resins, paints, dyes and plastics; its production is increasing every year. Therefore, environmental pollution by phenol and its effect on living organisms have come into question. It readily permeates through the skin and mucous membrane and is also quickly absorbed from the gastroenteric tract. As to the toxicity of phenol, local damage due to its contact with the skin and damage to various organs caused by oral or subcutaneous administration to animals have been reported (1-3).

Monohalogenated phenols such as fluorophenol (FPh), chlorophenol (ClPh), bromophenol (BrPh) and iodophenol (IPh) are also used extensively for various organic synthetic processes, and a considerable number of reports have described the environmental movement, biological concentration and toxicity of these compounds (1, 4-7).

As to the mode of toxic action of phenolic compounds, 2,4-dinitrophenol (DNP) and pentachlorophenol (PCP) are well-known as uncouplers of oxidative phosphorylation (8-10). Ogata *et al.* (11) classified various

*To whom correspondence should be addressed.

Abbreviations used: Ph, phenol; FPh, fluorophenol; ClPh, chlorophenol; BrPh, bromophenol; IPh, iodophenol; PCP, pentachlorophenol; DNP, 2,4-dinitrophenol; RCI, respiratory control index

chemicals into four groups based on the combination of their effects on state 3 and state 4 respiration. According to this classification, phenolic compounds are included in the group of inhibitors having characteristics of both energy transfer inhibition and uncoupling. Hansch *et al.* (12), Fujita (13), Stockdale *et al.* (14) and Miyoshi *et al.* (15) have stated that the biological action of phenolic compounds correlates with their physicochemical properties such as hydrophobicity and electronic features. The toxic effect of phenolic compounds was studied in this investigation in relation to their physicochemical properties. This communication deals with the effect of monohalogenated phenols on rat liver mitochondria in correlation with Hammett's substituent constant (σ) (16) and the hydrophobic binding constant (π) (12).

Materials and Methods

Chemicals. Ph, *p*-FPh, *p*-ClPh, *p*-BrPh and *p*-IPh were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. ADP and ATP were from Sigma Chemical Co., St Louis, MO. Other chemicals were of the highest purity obtainable from commercial sources.

Mitochondrial preparations. Male Donryu rats weighing approximately 200 g and fed on a laboratory stock diet were fasted overnight and sacrificed by decapitation. Rat liver mitochondria were isolated according to the method of Utsumi (17), a modification of the method of Hogeboom *et al.* (18), and suspended in a medium containing 0.25 M sucrose and 4 mM Tris-HCl buffer (pH 7.5).

Measurement of respiratory activity. Mitochondria (1.1-1.9 mg protein/ml) were incubated in 3.5 ml of reaction medium containing 0.15 M KCl, 10 mM Tris-HCl buffer (pH 7.5) and 2.5 mM phosphate buffer (pH 7.5). The phenolic compounds were dissolved in absolute ethanol. Sodium succinate, as a respiratory substrate, was added to the reaction mixture to give a concentration of 5 mM, followed by addition of various concentrations of phenolic compounds, 0.3 mM ADP and 0.02 mM DNP at regular intervals. The incubation was carried out at 25°C with continuous stirring. Oxygen consumption was measured with a galvanic type oxygen electrode (Kyusui Kagaku Co., Tokyo) connected to a recorder. The respiratory control index (RCI) and ADP/O ratio were calculated as parameters of respiratory activity according to the method of Hagihara (19).

Measurement of K^+ release. Mitochondria (0.5-1.2 mg protein/ml) were incubated at 25°C with continuous stirring in 3 ml of reaction medium containing 0.15 M choline chloride and 10 mM Tris-HCl buffer (pH 7.5). The measurement of K^+ efflux was carried out with a glass K^+ electrode (Beckman) connected to a pH meter.

Measurement of ATPase activity. Mitochondria (0.9-1.4 mg protein/ml), 3.5 mM ATP and phenolic compounds were added to a medium containing 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$ and 5 mM Tris-HCl buffer (pH 7.4) to give a total volume of 2.0 ml. After incubation for 15 min at 25°C, 1ml of ice-cold 24% perchloric acid was added, and fully mixed. The mixture was allowed to stand for 10 min, and then the supernatant was separated by centrifugation at 3000 rpm for 1 min. Mitochondrial latent and DNP-stimulated ATPase activities were determined by measurement of liberated inorganic phosphate according to the method of Takahashi (20).

Determination of protein concentration. The mitochondrial protein concentration was determined by the biuret reaction (21) using bovine serum albumin as a standard.

Statistical analysis. As parameters of electronic and hydrophobic properties of *p*-halogenated phenols, Hammett's substituent constant (σ) and the hydrophobic binding constant (π) were used. Hammett's substituent constant σ is calculated according to the formula $\sigma = 1/\rho \log (K/K_0)$, where K is the rate constant of a substituted compound, K_0 that of the parent compound and ρ a reaction constant (16). The hydrophobic binding constant π is calculated according to the formula $\pi = \log (Px/Ph)$, where Px is the partition coefficient of a substituted compound between 1-octanol and water and Ph that of the parent compound. Regression analyses and multiple regression analyses were carried out between 50% inhibition of RCI and 50% release of K^+ by *p*-halogenated phenols and σ and π (12, 22).

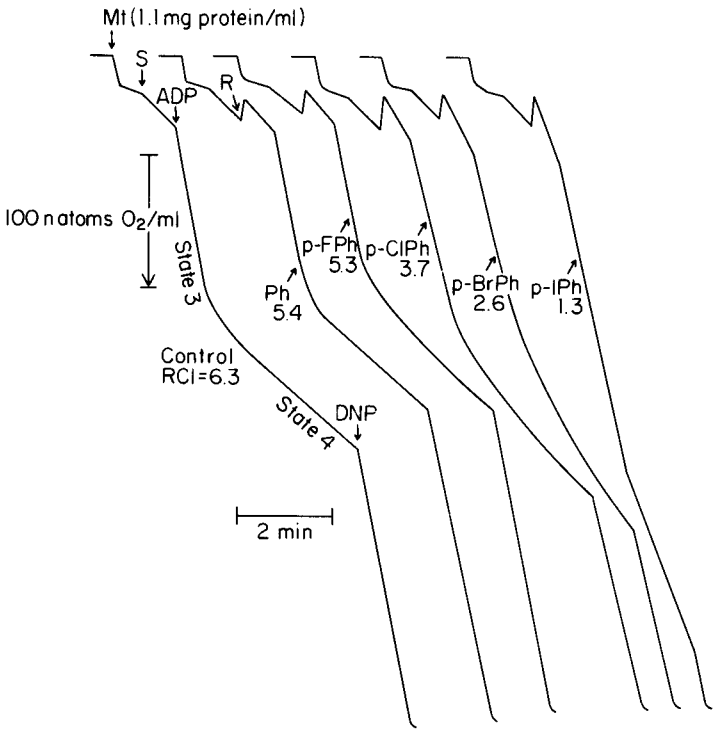

Fig. 1 Effect of phenol and *p*-halogenated phenols on mitochondrial respiration. Mitochondria (Mt, 1.1 mg protein/ml) were incubated in 3.5 ml of reaction medium containing 0.15 M KCl, 10 mM Tris-HCl buffer (pH 7.5) and 2.5 mM phosphate buffer at 25°C with continuous stirring. Succinate (S) (500 mM) was added as a substrate at 35 μl, followed, 30 sec later, by 30 mM phenols (R) dissolved in absolute ethanol, 30 mM ADP and 2 mM DNP at certain intervals. For abbreviations see footnotes.

Table 1 Effect of phenol and *p*-halogenated phenols on mitochondrial respiratory activity[a]

Compounds	State 3 respiration[b] (n atoms O_2/mg protein/min)	State 4 respiration[b] (n atoms O_2/mg protein/min)	RCI	ADP/O ratio
Control	113.2±3.2	18.4±2.9	6.2	2.2
Phenol (0.3 mM)	110.2±4.2	20.2±0.8	5.5	2.1
p-Fluorophenol (0.3 mM)	110.0±5.7	20.8±1.7	5.3	2.1
p-Chlorophenol (0.3 mM)	103.1±2.4	27.7±1.9	3.7	1.9
p-Bromophenol (0.3 mM)	94.5±3.2	37.0±4.4	2.6	1.6
p-Iodophenol (0.3 mM)	93.6±3.1	72.3±1.7	1.3	1.1

a: The experimental conditions were the same as those described in Fig. 1.
b: Values are the mean±S.D. of three determinations.

Table 2 Effects of phenol and *p*-halogenated phenols on various parameters of mitochondrial respiratory activity[a]

Compounds	Concentrations (moles/mg of protein) to induce:		
	50 % Inhibition of RCI	75 % of State 3 respiration	150 % of State 4 respiration
Phenol	5.0×10^{-6}	4.2×10^{-6}	6.5×10^{-6}
p-Fluorophenol	1.2×10^{-6}	1.5×10^{-6}	7.5×10^{-7}
p-Chlorophenol	2.5×10^{-7}	3.0×10^{-7}	2.0×10^{-7}
p-Bromophenol	2.0×10^{-7}	2.8×10^{-7}	1.5×10^{-7}
p-Iodophenol	1.1×10^{-7}	1.5×10^{-7}	0.9×10^{-7}

a: Experimental conditions were same as those described in Fig. 1.

Results

Effect of phenol and p-halogenated phenols on respiratory activities. Fig. 1 and Table 1 show the effects of the phenolic compounds (0.3 mM) on the respiratory activities of rat liver mitochondria. Phenol and *p*-halogenated phenols caused a slight decrease in state 3 respiration and a remarkable increase in state 4 respiration. The inhibitory effect of the phenolic compounds on RCI was in the descending order of *p*-IPh, *p*-BrPh, *p*-ClPh, *p*-FPh and Ph. The inhibition of RCI by the phenolic compounds was concentration-dependent. The concentrations of the phenolic compounds to cause 75% inhibition of state 3 respiration, 150% acceleration of state 4 respiration and 50% inhibition of RCI are summarized in Table 2. These data show that the inhibitory effect of these compounds on respiratory activities was in the same order of intensity in Table 2 as in Table 1.

Effect of phenols on ATPase activities. As shown in Fig. 2, the phenolic compounds stimulated latent ATPase activities as revealed by the acceleration of state 4 respiration. However, these compounds inhibited the DNP-stimulated ATPase activities at the same concentrations.

Effect of phenols on K^+ compartmentation. During the incubation of untreated control mitochondria, only a gradual K^+ efflux was seen. However, upon the addition of phenols, K^+ release was induced depending on the concentration of the phenols. The K^+-releasing effect of 1 mM *p*-halogenated phenols was in the descending order of *p*-IPh, *p*-BrPh, *p*-ClPh and *p*-FPh (Fig. 3). The concentrations of the phenolic compounds

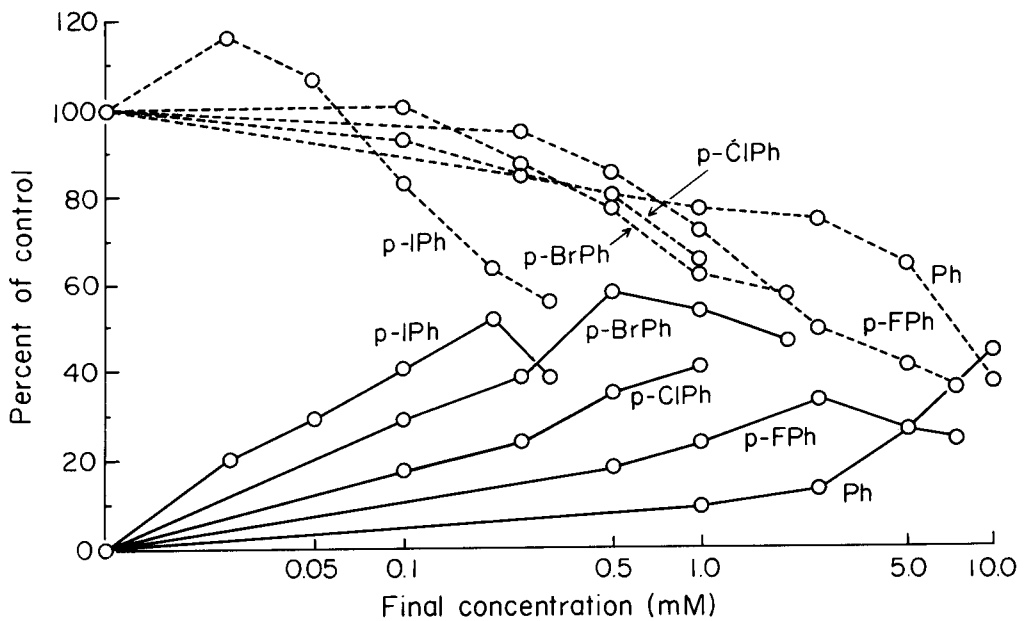

Fig. 2 Effect of phenol and p-halogenated phenols on the ATPase activities of mitochondria. Mitochondria (0.9-1.4 mg protein/ml), 3.5 mM ATP and varied concentrations of phenols were added to a medium containing 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$ and 5 mM Tris-HCl buffer (pH 7.4) to give a total volume of 2 ml. After incubation for 15 min at 25°C, 1 ml of ice-cold 24% perchloric acid was added, and after standing for 10 min, the supernatant was separated by centrifugation. The liberated inorganic phosphate was measured spectrophotometrically at 700 nm according to the method of Takahashi. The solid line and broken line indicate latent ATPase activity and DNP-stimulated ATPase activity, respectively. For abbreviations see footnotes.

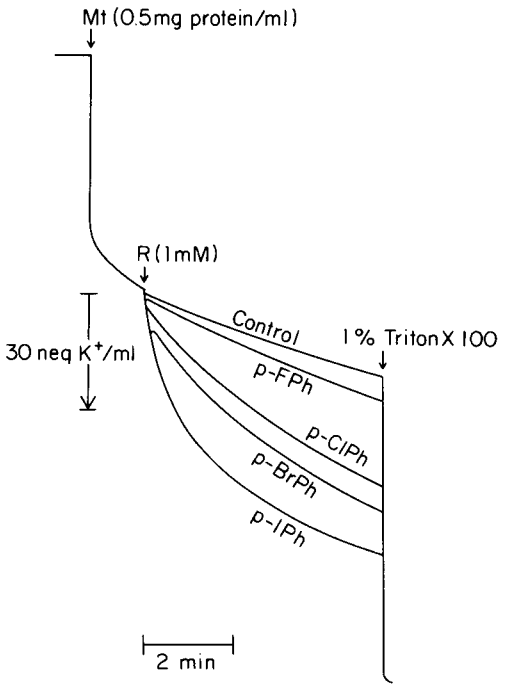

Fig. 3 Effect of *p*-halogenated phenols on the K^+ release of mitochondria. Mitochondria (0.5 mg protein/ml) were incubated in 3.0 ml of a reaction medium containing 0.15 M choline chloride and 10 mM Tris-HCl buffer (pH 7.5) at 25°C with continuous stirring. After incubation for 1.5 min, 30 μl of 100 mM phenols were added. Intramitochondrial content of K^+ ions was estimated by measuring the K^+ depleted by adding 1% Triton X. The measurement was carried out by using a glass K^+-selective electrode. For abbreviations see footnotes.

which induced a 50% release of K^+ were *p*-IPh, 1.75; *p*-BrPh, 4.0; *p*-ClPh, 4.8; *p*-FPh, 14, and Ph, 68 μmoles/mg protein (Fig. 4). These results coincided with the results of the respiratory activity study.

Correlations

Correlations of K^+-releasing effect and inhibition of RCI to Hammett's substituent constant σ. The plot of $\log(C/C_0)$ against σ, in which C stands for K^+ release (neq/mg protein/min) by each *p*-halogenated phenol and C_0 for K^+ release (neq/mg protein/min) by phenol is shown in Fig. 5. A high correlation was found between the K^+-releasing effect of the phenolic compounds and their Hammett's substituent constant (σ).

The relationship between 50% inhibition of RCI and σ values and that between 50% release of K^+ and σ values are shown in Fig. 6. Both 50% inhibition of RCI and 50% release of K^+ by the phenolic compounds correlated highly to σ values. The regression equation for the former is expressed as $\log(1/C) = 5.56\sigma + 5.41$ ($r = 0.97$) and that for the latter as $\log(1/C) = 4.79\sigma + 4.32$ ($r = 0.96$) ($p < 0.05$).

Relationship between 50% inhibition of RCI and 50% release of K^+ and the hydrophobic binding constant π. As shown in

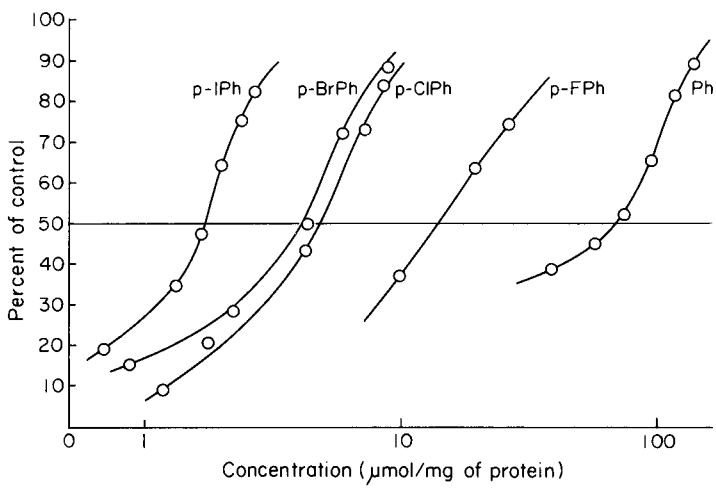

Fig. 4 K^+ release from mitochondria at various concentrations of phenol and *p*-halogenated phenols. The experimental conditions were the same as in Fig. 3. For abbreviations see footnotes.

Fig. 7, the equation for 50% inhibition of RCI against π is expressed as $\log(1/C) = 1.12\pi+5.44$ ($r = 0.97$), and that for 50% release of K^+ as $\log(1/C) = 0.98\pi+4.34$ ($r = 0.97$). In each case, there was a high correlation ($p < 0.05$).

Relationship between 50% inhibition of RCI and 50% release of K^+ and both σ and π of p-halogenated phenols. The multiple regression equation for 50% inhibition of RCI against both σ and π was obtained by least square analysis and expressed as $\log(1/C) = 3.62\sigma + 0.40\pi + 5.42$ ($r = 0.98$). Similarly, the relationship between 50% release of K^+ and both σ and π was expressed as $\log(1/C) = 0.88\sigma+0.80\pi+4.33$ ($r = 0.97$).

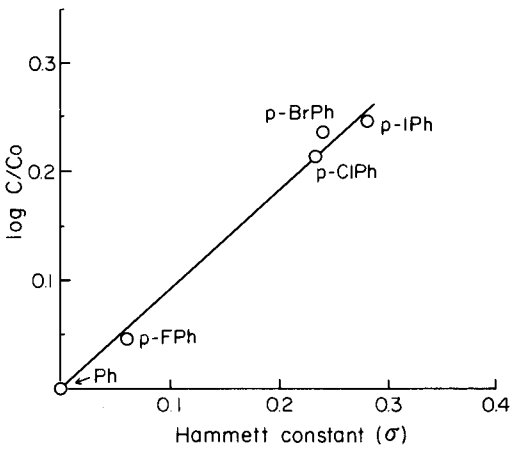

Fig. 5 Relationship between Hammett's substituent constant and $\log(C/C_0)$ of K^+ release by phenol and *p*-halogenated phenols.
C and C_0: neq K^+ released/mg of protein per min in the presence of *p*-halogenated phenols or phenol, respectively.

Discussion

The mechanism of the action of chemical compounds has been elucidated based on their chemical structure and physicochemical properties (23). It is proposed that the sterilizing action of phenolic compounds is strengthened by substitution with halogens, as seen in DNP whose uncoupling action is increased by substitution with a nitro group ($-NO_2$), an electronegative group (24).

A structure-activity relationship method has been developed by Hansch *et al.* (12) and Fujita (13) using substituted constants of phenols. Weinbach and Garbus (8) report-

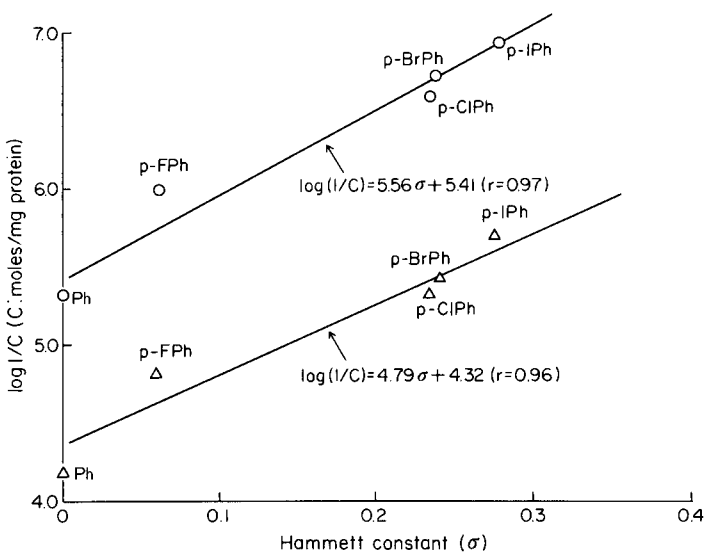

Fig. 6 Relationship between biological activities of *p*-halogenated phenols and Hammett's substituent constant.
The symbols ○ and △ indicate 50% inhibition of RCI and 50% release of K^+, respectively. For abbreviations see footnotes.

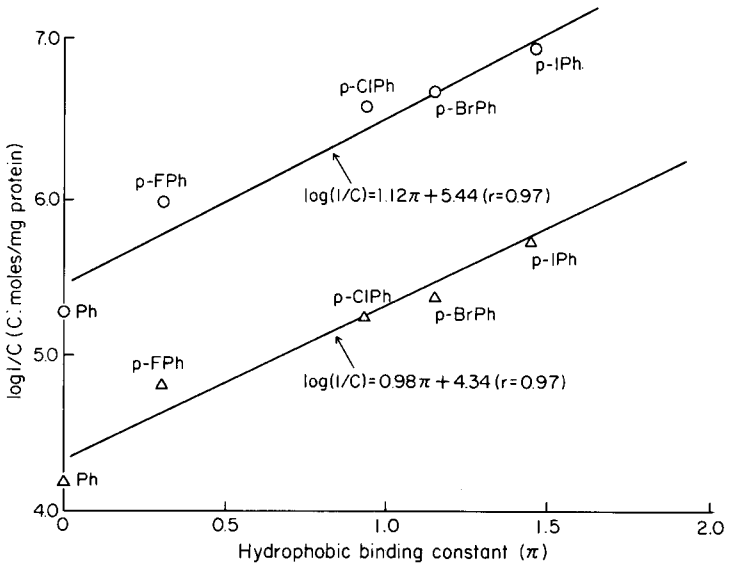

Fig. 7 Relationship between biological activities of *p*-halogenated phenols and hydrophobic binding constant. The symbols ○ and △ indicate 50% inhibition of RCI and 50% release of K^+, respectively. For abbreviations see footnotes.

ed the adsorption of phenols into mitochondrial protein and concluded that phenols reacted with the protein moiety of intact mitochondria to exert their uncoupling activity. Hansch *et al.* and Fujita analyzed the uncoupling activity of phenols with pKa and π using the data of Weinbach (8) and Hemker (25) and reported that lipophilic and electronic characteristics contribute to the uncoupling activity. Stockdale *et al.* (14) also reported a difference in the coefficients of σ and π between the correlation equation for uncoupling and that for inhibition of respiration by phenolic compounds: the coefficient of σ was larger than that of π in the correlation equation for uncoupling action, but the coefficient of σ was smaller than that of π in the correlation equation for inhibition of respiration.

A comparative examination of the mechanism of toxic effects of monohalogenated phenols has not been done. The induction of intramitochondrial K^+ release by phenols indicates that phenols accelerate membrane permeability to K^+ ions. The agreement in the order of the strength of action of these compounds on respiratory activities and on K^+-releasing activity indicates that a close correlation exists between membrane permeability and the energy transfer reaction of mitochondria.

There were significant correlations ($p < 0.05$) between the respiration-inhibiting activity of phenols and σ and π. Similar relationships existed between the K^+ release action of phenols and σ and π. The coefficients in the multiple regression equation were quite different from those in the correlation equations for inhibition of respiratory activity (3.62 for σ and 0.40 for π). This result indicates that the contribution of the electronic effect of the substituents is important in determinating the inhibition of respiration by halogenated phenols. The coefficients of 0.88 for σ and 0.80 for π in the equation of K^+ release are similar to each other. This fact indicates that the electronic property (expressed as σ) and hydrophobic property (expressed as π) contributed approximetely to the same degree. The "structure-activity relationship" analysis was shown to be useful in the present study for the evaluation of the toxic effect of halogenated phenols.

Acknowledgments. The authors thank Prof. Kazuhisa Taketa and Dr. Bunji Inoue for their suggestions and valuable discussion.

References

1. Deichmann WB and Keplinger ML: Phenols and phenolic compounds; in Industrial Hygiene and Toxicology, Vol 2, Patty ed, Interscience, New York (1981) pp 2567-2627.
2. Pharmacopoea Japonica. Vol XI, Hirokawa Publishing Co., Tokyo (1986) pp 818-823.
3. Aitio A, Riihimaki V and Vainio H: Biological Monitoring and Surveillance of Workers Exposed to Chemicals. Hemisphere Publishing Co., New York (1984) pp 171-172.
4. Exon JH: A review of chlorinated phenols. Vet Hum Toxicol (1984) **26**, 508-520.
5. Gebefügi L, Parlar H and Korte F: Occurrence of pentachlorophenol in enclosed environments. Ecotoxicol Environ Safety (1979) **3**, 269-300.
6. Paasivirta J, Särkka J, Leskijärvi T and Roos A: Transportation and enrichment of chlorinated phenolic compounds in different aquatic food chains. Chemosphere (1980) **9**, 441-456.
7. Morgade C, Barquet A and Pfaffenberger CD: Determination of polyhalogenated phenolic compounds in drinking water, human blood serum and adipose tissue. Bull Environ Contam Toxicol (1980) **24**, 257-264.
8. Weinbach EC and Garbus J: The interaction of uncoupling phenols with mitochondria and with mitochondrial protein. J Biol Chem (1965) **240**, 1811-1819.
9. Terada H and Muraoka S: Uncouplers. Tanpakusitu Kakusan Kouso (1973) **18**, 911-929 (in Japanese).
10. Heytler PG: Uncouplers of oxidative phosphorylation. Pharmacol Ther (1982) **10**, 461-472.
11. Ogata M, Mori T, Izushi F, Etoh K, Sakai R, Meguro T and Inoue B: Classification of potentially toxic chemicals based on thier effects on mitochondrial respiration. Physiol Chem Phys Med NMR (1983) **15**, 229-232.
12. Hansch C, Kiehs K and Lawrence GL: The role of substituents in the hydrophobic bonding of phenols by serum and mitochondrial proteins. J Am Chem Soc (1965) **87**, 5770-5773.
13. Fujita T: The analysis of physiological activity of substituted phenols with substituent constants. J Med Chem (1966) **9**, 797-804.
14. Stockdale M and Selwyn MJ: Effects of ring substituents on the activity of phenols as inhibitors and uncouplers of mitochondrial respiration. Eur J Biochem (1971) **21**, 565-574.
15. Miyoshi H, Nishioka T and Fujita T: Quantitative relationship between protonophoric and uncoupling activities of substituted phenols. Biochim Biophys Acta (1987) **891**, 194-204.
16. Jaffé H H: A reëxamination of the hammett equation. Chem Rev (1953) **53**, 191-254.
17. Utsumi K: Relation between mitochondrial swelling induced by inorganic phosphate and accumulation of ^{32}P in mitochondrial Pi fraction. Acta Med Okayama (1963) **17**, 259-271.
18. Hogeboom GH, Schneider WC and Pallade GE: Cytochemical studies of mammalian tissues. 1. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J Biol Chem (1948) **172**, 619-635.
19. Hagihara B: Technique for the application of polarography to mitochondrial respiration. Biochim Biophys Acta (1961) **46**, 134-142.
20. Takahashi T: Determination of true inorganic phosphate and creatine phosphate in tissues and on the phosphoamidase and creative phosphokinase of swine spermatozoa. J Jpn Biochem Soc (1955) **26**, 690-698 (in Japanese).
21. Gornall AG, Bardawill CJ and David MM: Determination of serum proteins by the biuret reaction. J Biol Chem (1949) **177**, 751-766.
22. Tanoue A: Partition coefficient and solubility of chemicals. Seitaikagaku (1982) **5**, 48-61 (in Japanese).
23. Terada H and Fujita T: Yakubutusayo no Kozokasseisoukan. Hirokawa Publishing Co., Tokyo (1974) pp 1-72 (in Japanese).
24. Parker VH: Effect of nitrophenols and halogenophenols on the enzymic activity of rat liver mitochondria. Biochem J (1958) **69**, 306-311.
25. Hemker HC: Lipid solubility as a factor influencing the activity of uncoupling phenols. Biochim Biophys Acta (1962) **63**, 46-54.

Received June 1, 1987; accepted October 27, 1987