Development of QSARs for Predicting the Joint Effects between Cyanogenic Toxicants and Aldehydes

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Quantitative structure−activity relationship (QSAR) approaches are proposed in this study to predict the joint effects of mixture toxicity. The initial investigation studies the joint effects between cyanogenic toxicants and aldehydes to *Photobacterium phosphoreum*. Joint effects are found to result from the formation of a carbanion intermediate produced through the chemical interactions between cyanogenic toxicants and aldehydes. Further research indicates that the formation of carbanion intermediate is highly correlated with not only the charge of the carbon atom in the −CHO of aldehydes but also the charge of the carbon atom (C*) in the carbochain of cyanogenic toxicants. The charge of the carbon atom in the −CHO of aldehydes is quantified by using the Hammett constant (σ_p), and then, σ_p-based QSAR models are proposed to describe the relationships between the joint effects and the chemical structures of the aldehydes. By using the charge of carbon atom (C*) in the carbochain of cyanogenic toxicants, another QSAR model is proposed to describe the relationship between the joint effects and the chemical structures of cyanogenic toxicants.

Introduction

It has been widely recognized that aquatic organisms in the environment are usually exposed to multiple rather than to single chemicals, and prediction of mixture toxicity is becoming an area of intense research interest to environmental scientists. However, most studies have focused on developing the rational methodologies to classify joint effects, whereas few studies have focused on predicting the joint effects of mixture toxicity (*1*, *2*). A general approach for predicting joint effects of mixture toxicity is therefore necessary and will improve risk assessment and ecological criteria establishment.

Introduced by pharmacologists, the quantitative structure−activity relationship (QSAR) approach, based on the mathematical relationship between chemical structure and toxicity, has emerged as one of the most effective methods to predict the toxicity of single organic chemicals. If this mathematical relationship could be extended to mixtures, QSAR approaches could be developed in the field of the mixture; therefore, the joint effects of mixture toxicity could be predicted by this approach. However, without a solid understanding of the interactions of individual chemicals in the organism, the interaction-based mechanism underlying mixture toxicity could not be revealed explicitly; furthermore, the mechanism-based mathematical relationship could not be obtained. In conclusion, the major bottleneck to development of QSARs for mixtures is the limited understanding of relevant chemical interactions within mixtures in relation to biological effects.

In 1996, Chen (*2*) studied a mixture toxicity using Microtox tests and observed the synergistic effect between malononitrile and aldehydes (formaldehyde, acrolein, and acetaldehyde). It was suggested in that research that the occurrence of such a synergistic effect was caused by the formation of more toxic products through chemical interactions between the two parent chemicals in the mixture. However, the exact interactions in organisms were not fully revealed in any of his further research work. Meanwhile, Shinkai (*3*) observed that in the presence of cyanide ion aldehydes undergo conversion to carbanion intermediates, which can be readily oxidized to the corresponding carboxylic acids or converted to benzoin condensations. If the interactions between malononitrile and aldehydes in *Photobacterium phosphoreum* are identical with those observed by Shinkai (*3*) from a pure chemistry viewpoint, Shinkai's method, after some necessary modification, therefore could be used to describe the interactions between malononitrile and aldehydes in *P. phosphoreum*.

The purpose of this study is as follows: first, to determine the joint effects between cyanogenic toxicants and aldehydes to *P. phosphoreum*; second, to study the interactions between individual chemicals in organisms; third, to reveal the mechanism of joint effects between cyanogenic toxicants and aldehydes; fourth, to analyze the relationship between the joint effects and the individual chemical structures; and finally, to further develop QSAR approaches for predicting joint effects of mixture toxicity.

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Table 1. Results of the Individual Toxicity Experiment

	individual toxicants	molecular formula	$-\log EC_{50}$ (mol L^{-1})
cyanogenic toxicants	malononitrile	$CH_2(CN)_2$	2.55
	α-hydroxy-isobutyro-nitrile	$(CH_3)_2C(OH)CN$	3.61
	acrylonitrile	$H_2C{=}CHCH_2CN$	1.45
	acetonitrile	C_2H_5CN	2.22
	salicylonitrile	$C_6H_4(OH)CN$	3.51
	benzonitrile	C_6H_5CN	3.48
	phenylacetonitrile	$C_6H_5(CH_2)_2CN$	4.23
aldehydes	acetaldehyde	CH_3CHO	2.36
	n-propylaldehyde	C_2H_5CHO	2.72
	n-butylaldehyde	C_3H_7CHO	3.04
	n-valeraldehyde	C_4H_9CHO	3.50
	n-enanthaldehyde	$C_6H_{13}CHO$	4.05
	n-capraldehyde	$C_9H_{19}CHO$	4.47
	p-nitrobenzaldehyde	$NO_2(C_6H_4)CHO$	4.28
	terephthalic aldehyde	$CHO(C_6H_4)CHO$	4.31
	p-chlorobenzaldehyde	$Cl(C_6H_4)CHO$	4.25
	p-bromobenzaldehyde	$Br(C_6H_4)CHO$	4.35
	benzaldehyde	C_6H_5CHO	3.43
	p-methylbenzaldehyde	$CH_3(C_6H_4)CHO$	3.68
	p-methoxybenzaldehyde	$CH_3O(C_6H_4)CHO$	4.03
	p-hydroxybenzaldehyde	$HO(C_6H_4)CHO$	4.37

Table 2. Results of the Joint Effects between Malononitrile and Individual Aldehydes

individual aldehydes	*M*	Hammett constant[a] substituent	σ_p
nitrobenzaldehyde	0.228	NO_2	0.78
terephthalic aldehyde	0.176	CHO	0.42
p-chlorbenzaldehyde	0.632	Cl	0.23
p-bromobenzaldehyde	0.688	Br	0.21
benzaldehyde	0.682	H	0.00
p-methylbenzaldehyde	0.928	CH_3	−0.16
p-methoxybenzaldehyde	1.072	OCH_3	−0.27
p-hydroxybenzaldehyde	1.154	OH	−0.37
acetaldehyde	0.115	H	0[b]
n-propyl aldehyde	0.123	CH_3	−0.03[b]
n-butyl aldehyde	0.160	C_2H_5	−0.06[b]
n-valeric aldehyde	0.308	C_3H_7	−0.07[b]
n-enanthic aldehyde	0.764	C_5H_{11}	−0.14[b]
n-capric aldehyde	0.782	C_8H_{17}	−0.16[b]

[a] Note: the Hammett constants were obtained from Hansch (*20*). [b] Note: according to Alan (*21*), the Hammett constants were calculated as follows: $(\sigma_i)_X = [(pK_a)_{CH_3CO_2H} - (pK_a)_{X-CH_2CO_2H}]/3.95$, in which pK_a was obtained from literature (*22*).

Experimental Procedures

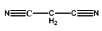
Materials. Seven cyanogenic toxicants and 14 aldehydes (Table 1) were purchased in the highest purity available from ACROS Organic Inc. The purity of aromatic aldehydes was monitored by HPLC to ensure that there was no interference peak. All mixtures were tested in equitoxic doses (identical fractions of EC_{50}) based on observed EC_{50} values. The compositions of mixtures are given in Tables 2 and 3. The *p*-nitrobenzoic acid, *p*-phthalic acid, *p*-chlorbenzoic acid, *p*-hydroxybenzoic acid, benzoic acid, CTMAB (hexadecyltrimethylammonium bromide), KCN, and HCl were analytical reagent grade.

Instrumentation. A Waters 600 HPLC, with a Waters 996 photodiode array detector, was employed for analysis of product acids. The toxicity test instrument (model toxicity analyzer DXY-2) was made by the Institute of Soil Science, Academic Sciences, Nanjing, P.R.C.

The freeze-dried marine bacterium, *P. phosphoreum* (T_3 mutation), was supplied by the Institute of Soil Science, Academic Sciences. It was reconstituted and maintained on agar slants at 4 °C. The bioluminescence assays were performed using the diluted bacteria that had been cultured at 20 °C in yeast−tryptone−salts−gycerol broth for 12−14 h.

Chemical Interactions between Cyanogenic Toxicants and Aldehydes. According to Shinkai (*3*), this reaction was initiated by mixing 10 mL of malononitrile (15 mM or 10 mL of KCN (7.5 mM)), 5 mL of aldehydes (0.1 mM), and 40 mL of CTMAB (2.0 mM) in a 250 mL glass bottle with screw caps. This mixture solution was kept at 30 ± 0.5 °C by immersing the bottle in a water bath for 24 h and then was acidified with 4 N HCl to pH 2.50. Finally, this acidified solution was filtrated with the 0.45 μm water film and analyzed by HPLC.

Table 3. Results of the Joint Effects between Acetaldehyde and Individual Cyanogenic Toxicants

individual cyanogenic toxicants	possible carbon atom (C*) in the carbochain of cyanogenic toxicants		M[b]
	Position (C*)	Charge[a]	
malononitrile		-0.086	0.11
α -hydroxy-isobutyronitrile		-0.075	0.62
acetonitrile		-0.259	1.06
acrylonitrile		-0.161	1.35
salicylonitrile		-0.178	1.19
benzonitrile		-0.171	1.00
phenylacetonitrile		-0.121	0.93

[a] The charge of C* was calculated using the MOPAC (version 6.0) program (*18*). [b] The values of *M* were calculated using eq 1.

Product Analysis. The acids were analyzed by using HPLC, and their yields were determined by comparing the integrated intensity of the peaks with that of authentic samples. HPLC was performed with a SC-100, Kromasil C_{18} column (200 mm × 4.6 mm, 5 μm) under mobile phase ($V_{methanol}$:V_{water} = 50:50, acidified with 4 N HCl to pH 2.50) at a flow rate of 0.8 mL/min.

Toxicity Experiment. Toxicity was measured with DXY-2 by quantifying the decrease in light emission from the bacteria as a result of exposure to the test chemicals in 3% NaCl medium solution for 15 min. The decrease in light emission was measured at six different concentrations, and each was tested in triplicate. On the basis of the decrease in light emission, median effective concentration (EC_{50}) was calculated using the probit model (*4*) and reported as $lg1/EC_{50}$ in units of mol L^{-1}. The toxicity of 21 single chemicals was observed, and the results are listed in Table 1. The mixture toxicity was conducted in a similar manner as the single chemicals tests. The joint effects are presented by the sum of toxic units (*M*, eq 1) rather than the traditional isobogram analyses, since the results of these two methods are equivalent for equitoxic mixtures (*5*).

$$M = \frac{z_1}{Z_1} + \frac{z_2}{Z_2} \quad (1)$$

where z_i is the toxicant concentration and Z_i is the EC_{50} value. The combination of z_1 and z_2 results in an exact 50% response. Simple addition is characterized by $M = 1$, where $M > 1$ represents antagonism and $M < 1$ indicates synergism. The *M* of mixtures is given (Tables 2 and 3).

Statistical Analyses. Statistical analyses were performed using the SPSS 9.0 software (SPSS Inc.). The coefficient of determination (r^2), standard error (SE), F ratio, and P value were taken into consideration in testing the quality of the regression.

Results and Discussion

Joint Effects of Mixture Toxicity. Before measuring the joint effects of mixture toxicity, the toxicity (EC_{50}) of 21 individual chemicals to *P. phosphoreum* was observed (Table 1). On the basis of these observed EC_{50} values, the joint effects between malononitrile and individual aldehydes were determined and the results are listed in Table 2. As shown in Table 2 for the mixtures containing malononitrile and aldehydes, the joint effects are different (synergistic or additive), and this difference depends on the individual aldehydes: $M_{\text{terephthalic aldehyde}} > M_{p-\text{nitrobenzaldehyde}} > M_{p-\text{chlorbenzaldehyde}} > M_{p-\text{bromobenzaldehyde}} > M_{\text{benzaldehyde}} > M_{p-\text{methylbenzaldehyde}} > M_{p-\text{methoxybenzaldehyde}} > M_{p-\text{hydroxybenzaldehyde}}$.

Meanwhile, the joint effects between acetaldehyde and cyanogenic toxicants were also determined (Table 3). It can be seen from Table 3 that the toxicity of acetaldehyde is synergistic ($M < 1$) with either malononitrile or α-hydroxy-isobutyronitrile, and acetaldehyde interacts via the antagonistic mode with acetonitrile ($M > 1$), whereas the additive effect ($M \approx 1$) is found in mixtures containing acetaldehyde and other cyanogenic toxicants.

As indicated by the bioluminescent reaction of *P. phosphoreum* in eq 2, the long chain aldehyde (RCHO) is oxidized in the presence of bacterial luciferase as the catalyzer.

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferas}} FMN + H_2O + RCOOH + \text{light} \quad (2)$$

It can be seen from eq 2 that the long chain aldehyde (RCHO) is the substrate for luciferase in this reaction (*6*, *7*). This leads to the assumption that for mixtures containing toxicant aldehydes and malononitrile, if the toxicant aldehydes could compete against the long chain aldehyde for substrate sites, malononitrile would induce luciferase to bind the toxicant aldehydes. Therefore, the toxicant aldehydes inhibit luciferase from the normal bioluminescent reaction (eq 2), and then, this leads to the synergistic toxicity between toxicant aldehydes and malononitrile. However, two independent aldehyde-binding sites, with a higher affinity site for the aldehyde substrate (long chain aldehyde) and a weaker affinity site for the aldehyde inhibitor (toxicant aldehyde), were detected by Lei (*8*). This, of course, refuted the assumption mentioned above. Therefore, to find the mechanism of the joint effects, Chen's (*2*) proposal about chemical interactions between malononitrile and aldehydes in organisms (see Introduction) should be taken into serious consideration.

Chemical Interactions between Cyanogenic Toxicants and Aldehydes. In 1980, Shinkai (*3*) found that in the presence of cyanide ion as catalyzer, aldehydes usually undergo conversion to the transient carbanion intermediates, which are easily oxidized by flavin (FMN) to the corresponding carboxylic acids. To study the chemical interactions between malononitrile and aldehydes in *P. phosphoreum*, Shinkai's method is modified as follows.

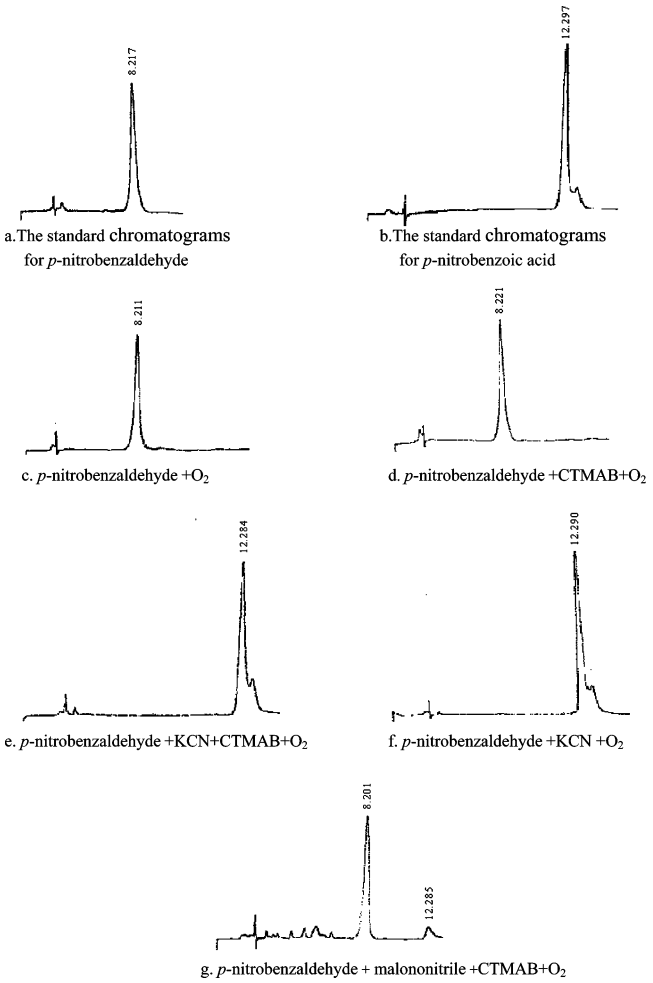


Figure 1. HPLC results of oxidation reaction of *p*-nitrobenzaldehyde in the presence of CN^- or malononitrile. The oxidation reaction was maintained at 30 ± 0.5 °C for 24 h; (a) acrylonitrile and (b) malononitrile.

1. O_2 Takes the Place of FMN as the Oxidant. As indicated by eq 3, O_2 is a more effective oxidant than FMN (*9*, *10*).

$$FMNH_2 + O_2 \rightarrow FMN + H_2O_2 \quad (3)$$

Therefore, in the presence of cyanide ion (KCN) and cationic micelle (CTMAB), *p*-nitrobenzaldehyde is oxidized by O_2 in this study instead of by FMN in the study of Shinkai (*3*). The same reaction is repeated without O_2 (under N_2 stream). The yields of product (*p*-nitrobenzoic acid) for these two reactions are 87.4 and 0%, respectively. This result, similar to that observed by Shinkai (*3*) in the case of *p*-chlorobenzaldehyde, indicates that O_2 is an excellent oxidant for the carbanion intermediates.

2. Malononitrile vs CN^-. In 1971, Goodman (*11*) observed that the reactivity of cyanoethyl in cyanoethylene is identical with that of CN^-. Obviously, it will lead to our assumption that cyanoethyl in malononitrile carries the same catalysis as CN^- in the oxidation process of *p*-nitrobenzaldehyde. This assumption is proved to be valid in Figure 1. As can be seen from Figure 1, no oxidation occurs in the absence of malononitrile or CN^- (Figure 1c,d), but the oxidation product (*p*-nitrobenzoic acid) will be yielded once there is CN^- or malononitrile (Figure 1e−g). This indicates that similar to CN^-, malononitrile is able to act as catalyzer to facilitate the

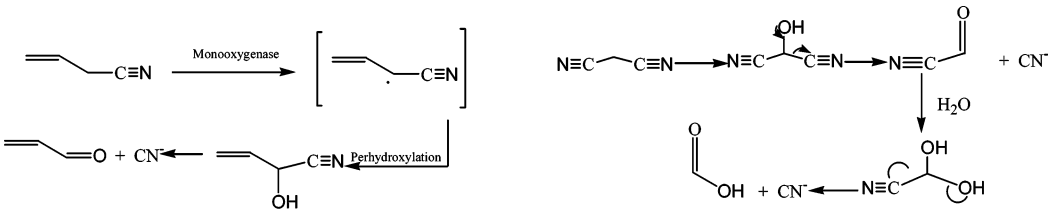


Figure 2. Scheme illustrating generation of cyanide from acrylonitrile (a) and malononitrile (b) via monooxygenase activation (after Lipnick (*12*)).

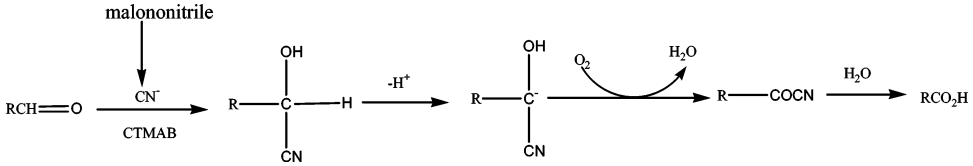


Figure 3. Chemical mechanism of joint effect between malononitrile and aldehyde: the interaction between cyanide ion and aldehyde results in the formation of carbanion intermediate, and this carbanion intermediate can be easily oxidized by O_2 to the corresponding acid.

Table 4. Yield of Acids from Six Aldehydes by Oxidation Reaction

	yield = acid/aldehyde × 100%			
	catalyzer			
	KCN		malononitrile	
	CTMAB (mM)			
aldehydes	2.0	0	2.0	0
terephthalic aldehyde	108.9	67.7	11.2	0
p-nitrobenzaldehyde	94.0	87.4	7.2	0
p-chlorobenzaldehyde	19.2	0	0	0
benzaldehyde	1.2	0	0	0
p-hydroxybenzaldehyde	0	0	0	0
p-methoxybenzaldehyde	0	0	0	0

formation of the carbanion, although the catalytic activity of malononitrile is found to be relatively low in comparison of the yield of *p*-nitrobenzoic acid in Figure 1e (84%) with that in Figure 1g (7.2%). Furthermore, even if malononitrile has no catalysis, it can be used as "catalyzer" in the chemical interaction between malononitrile and *p*-nitrobenzaldehyde in *P. phosphoreum*, as Lipnick (*12*) found that malononitrile can generate CN^- physiologically (Figure 2b).

3. Effect of CTMAB Micelle. To study the effect of CTMAB micelle, the oxidation of *p*-nitrobenzaldehyde, terephthalic aldehyde, and *p*-chlorobenzaldehyde in the nonmicellar system is compared with that in the presence of CTMAB micelle (Table 4). As shown in Table 4, the introduction of CTMAB remarkably increases the yield of acids. Because CTMAB is a cationic surfactant, it can concentrate the cyanide ion (CN^-) to CTMAB by the Coulomb interaction and can attach the aldehyde to CTMAB by a hydrophobic interaction (*3*). Therefore, the CTMAB micelle enhances the collision probability between the CN^- and the aldehyde, thus facilitating the formation of carbanion intermediate.

At last, according to the findings mentioned above, the chemical interaction between malononitrile and *p*-nitrobenzaldehyde in *P. phosphoreum* is proposed in Figure 3. As can be seen in Figure 3, in the presence of CTMAB and CN^- (or malononitrile), aldehydes undergo conversion to the transient carbanion intermediates, which are easily oxidized by O_2 to the corresponding carboxylic

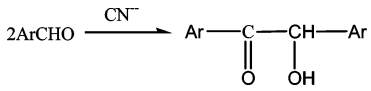


Figure 4. Aromatic aldehydes undergo benzoin condensation in the presence of cyanide ion (after Ide (*13*) and Finer (*14*)).

acids. Then, the oxidation of six aldehydes takes place and the results are listed in Table 4. It can be seen from Table 4 that according to the degree of conversion to the corresponding acids, the aldehydes can be ranked as follows: terephthalic aldehyde > *p*-nitrobenzaldehyde > *p*-chlorbenzaldehyde > benzaldehyde > *p*-methoxybenzaldehyde ≈ *p*-hydroxybenzaldehyde. This rank is identical with that of *M* in Table 2: the greater the conversion, the stronger the synergistic toxicity, and zero conversion corresponds to the additive effect. This identity supports our proposal in Figure 3.

Chemical Mechanism of Joint Effects. As mentioned above, the chemical interactions between malononitrile and aldehydes lead to the transient carbanion intermediates. These intermediates are then easily oxidized by O_2 to the corresponding carboxylic acids. In addition, as shown in Figure 4, Ide (*13*) and Finer (*14*) observed that these intermediates can also be converted to benzoin condensations. This indicates that the joint effects toxicity will arise from either carboxylic acids or benzoin condensations.

1. Impact of Carboxylic Acids on the Joint Effects of Mixture Toxicity. Baterle (*16*) pointed out that some particular aromatic aldehydes with a strong electron-withdrawing substituent (e.g., nitro) in the *p*- or *o*-position could not undergo benzoin condensation. This viewpoint is confirmed in Figure 1e: in the presence of cyanide ion, *p*-nitrobenzaldehyde only yields *p*-nitrobenzoic acid but not benzoin condensation. Therefore, *p*-nitrobenzaldehyde is an ideal research subject to study the impact of carboxylic acids on the joint effects toxicity, as it will not be converted to benzoin condensation. The effect of *p*-nitrobenzoic acid on the joint effects is listed in Table 5. It can be seen from Table 5 that *p*-nitrobenzoic acid (no. 2, $1/\log EC_{50} = 3.48$ mol L^{-1}) has a lower toxicity than *p*-nitrobenzaldehyde (no. 1, $1/\log EC_{50} = 4.28$ mol L^{-1}). Furthermore, according to the *M* (0.95−1.12) of nos. 3, 4, and 6, *p*-nitrobenzoic acid only has the additive effect

Table 5. Effect of Product *p*-Nitrobenzoic Acid on the Mixture Containing Malononitrile and *p*-Nitrobenzaldehyde

no.	individual chemicals	toxicity
1	*p*-nitrobenzaldehyde	$1/\log EC_{50} = 4.28$ mol L^{-1}
2	*p*-nitrobenzoic acid	$1/\log EC_{50} = 3.48$ mol L^{-1}
3	*p*-nitrobenzoic acid + malononitrile ($2EC_{50}$:$2EC_{50}$)	$M = 1.12$ additive
4	*p*-nitrobenzoic acid + *p*-nitrobenzaldehyde ($2EC_{50}$:$2EC_{50}$)	$M = 0.99$ additive
5	malononitrile + *p*-nitrobenzaldehyde ($2EC_{50}$:$2EC_{50}$)	$M = 0.23$ synergistic
6	*p*-nitrobenzoic acid + no. 5 ($2EC_{50}$:$2EC_{50}$)	$M = 0.95$ additive

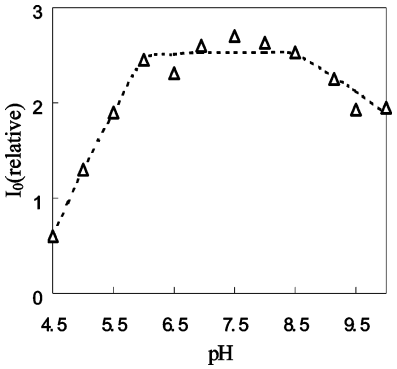
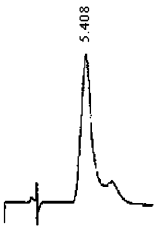
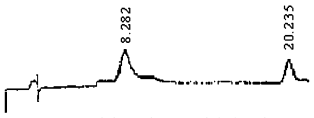


Figure 5. Stability of luciferase against pH. Enzyme was treated at each pH (in the absence of *n*-decylealdehyde) for 10 min at 0 °C. I_0 values were determined at pH 7.0 and 20.5 °C (after Nakamura (*15*)).

a. The standard chromatograms for *p*-chlorobenzaldehyde



b. The standard chromatograms for *p*-chlorobenzoic acid



c. *p*-chlorobenzaldehyde +KCN +O_2

Figure 6. HPLC results of the *p*-chlorobenzaldehyde experiment. The experiment was maintained at 30 ± 0.5 °C for 24 h.

for malononitrile, *p*-nitrobenzaldehyde, or for a mixture of the two. At first sight, it appears that the formation of *p*-nitrobenzoic acid does not lead to the synergistic effect between malononitrile and *p*-nitrobenzaldehyde. However, this conclusion was refuted by Nakamura (*15*). By using the absolute light intensity (I_0) to measure the enzyme activity, he observed that luciferase is stable during pH 6−8.5, and outside this pH range, any change of pH will depresses the enzyme activity (Figure 5). Therefore, being oxidized from *p*-nitrobenzaldehyde in the inhibitor-binding site/α*β*-luciferase (*8*), the product

Table 6. Effects of Benzoin Condensations on the Joint Effects between *p*-Chlorobenzaldehyde and CN^-

Reaction	Toxicity Test Solution	Decrease in Light Emission (%)
No.1: 30ppm KCN ($2EC_{50}$) $\xrightarrow{30\pm0.5\,^\circ\mathrm{C},\ 24\mathrm{h}}$	Assumed No.1 as 100%	60.0
No.2: 9.34ppm p-chlorobenzaldehyde $\xrightarrow{30\pm0.5\,^\circ\mathrm{C},\ 24\mathrm{h}}$	Assumed No.2 as 100%	90.1
No.3: 9.34ppm p-chlorobenzaldehyde +30ppmKCN $\xrightarrow{30\pm0.5\,^\circ\mathrm{C},\ 24\mathrm{h}}$ benzoin condensation	5mL No.3 + 5mL H_2O } 100%	89.6
No.4: No.1+ No. 2 room temperate, mix together immediately	5mL No.1 + 5mL No.2 } 100%	39.2

p-nitrobenzoic acid is bound in the inhibitor-binding site/α*β*-luciferase as well. The acid is in such close proximity to luciferase that it decreases the circumstance pH of luciferase remarkably. Because it has great efficiency in inhibiting enzyme activity, it further leads to the synergism between malononistrile and aldehydes.

2. Impact of Benzoin Condensation on the Joint Effects. In this study, the *p*-chlorbenzaldehyde experiment is carried out without CTMAB and the results are given in Figure 6. As shown in Figure 6c, there is no peak at $t = 5.408$ min, which denotes no yield of *p*-chlorobenzoic acid. However, two remarkable peaks are recorded at $t = 8.282$ min and $t = 20.235$ min. The same result was observed by Shinkai (*3*), and he further identified these two peaks as 4,4-dichlorobenzoin and 4,4′-dichlorobenzil by comparing the peaks with that of the authentic samples. This indicates that without CTMAB, *p*-chlorbenzaldehyde can only be converted to benzoin condensations but not *p*-nitrobenzoic acid. Therefore, *p*-chlorbenzaldehyde is an ideal research subject to study the impact of benzoin condensations on the joint effects. The effects of benzoin condensations on the joint effects are listed in Table 6. The comparison of nos. 1 and 2 with no. 3 in Table 6 indicates that the yields of benzoin condensations do not facilitate the light emission decreases; that is, the yields of benzoin condensations do not increase the toxicity of two parent chemicals (CN^- and *p*-nitrobenzaldehyde). This indicates that the synergistic effect between CN^- and *p*-nitrobenzaldehyde, shown by no. 4, does not result from the benzoin condensations.

In conclusion, the chemical mechanism of joint effects between malononistrile and aldehydes is revealed as follows: in the presence of malononistrile or CN^-, the aldehyde undergoes conversion to the carbanion inter-

mediate, and then, this intermediate is oxidized by O_2 to the corresponding acid. First, this intermediate traps O_2, thus inhibiting the bioluminescent reaction (eq 2, which indicates that O_2 is a prerequisite oxidant in the reaction) and other metabolism (such as respiration) of *P. phosphoreum*. Second, the acid might increase the effective acidity of luciferase and therefore inhibit the luciferase activity. Last, in the absence of O_2, the carbanion intermediate can be oxidized by FMN (*3*), thereby interrupting the physiological conversion of FMM to $FMNH_2$ in *P. phosphoreum* (eqs 4 and 5).

$$NAD(P) + RH_2 \xrightarrow{\text{dehydrase}} NADPH_2 + R \qquad (4)$$

$$NAD(P)H_2 + FMN - NAD(P) + FMNH_2 \qquad (5)$$

This mechanism indicates that the joint effects are closely related to the formation of carbanion intermediate. Therefore, if this formation could be studied from the prospect of the individual chemical structures, the relationships between the joint effects and the individual chemical structures could be elucidated.

Relationships between the Joint Effects and the Chemical Structures of Aldehyde. As shown in Figure 3, during the formation of the carbanion intermediate, cyanide ion acts as a nucleophile to attack the positively charged carbon atom in the −CHO group. The charge of the carbon atom in the aldehydic −CHO group therefore is expected to be highly correlated with the formation of the carbanion intermediate: the more positively charged the carbon atom in the −CHO group is, the easier cyanide ion attacks carbon atom, the easier the carbanion intermediate forms. The charge of a carbon atom largely depends on molecular context. For example, a positive charge will be induced on the carbon atom of the −CHO group if an electron-withdrawing substituent (e.g., NO_2) is introduced into the *p*-position of aromatic aldehydes. Similarly, for aliphatic saturated aldehydes, the charge of carbon atom in the α-C−H depends on the steric hindrance effect from the electron donation of the α-C saturated alkyl (*17*). In other words, the charge density of the carbon atom in the −CHO group can be quantified on condition that the electron-donating/electron-withdrawing ability of other groups in chemical structures can be evaluated. It is well-known that the electron-donating/electron-withdrawing ability of groups can be evaluated efficiently by the Hammett constant (σ_p). Therefore, for individual aldehydes, we propose the following correlation scheme between the σ_p and the joint effects.

joint effect ← formation of the carbanion ← charge density of carbon atom in CHO group ← chemical structure of individual aldehydes (σ_p)

The σ_p of individual aldehydes is listed in Table 2. Using these values of σ_p, a linear regression on the joint effects (M) is obtained

$$M = 0.577 - 0.691\sigma_p \qquad (6)$$

where $n = 14$, $r^2 = 0.316$, SE = 0.315, $F = 5.571$, and $P = 0.036$. However, only a poor correlation coefficient ($r^2 = 0.316$) is obtained in eq 6. To reveal the contributors to this poor correlation, the relation between σ_p and M is plotted in Figure 7. It can be seen from Figure 7 that

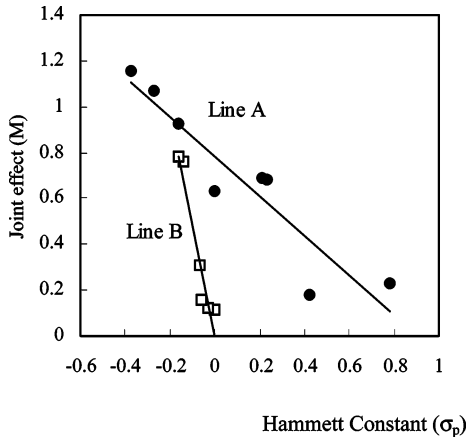


Figure 7. Relationship between the joint effects (M) and the Hammett Constant (σ_p) of individual aldehydes. Line A, eq 7 for the aromatic aldehydes; line B, eq 8 for the aliphatic aldehydes.

the relationship between σ_p and M for aliphatic aldehydes differs from that for the aromatic aldehydes. It is this difference that contributes to the poor correlation in eq 6. As shown in Table 2, this difference may result from the different calculation of σ_p between aliphatic aldehydes and aromatic aldehydes: the σ_p of aliphatic aldehydes is obtained based on the pK_a of acetic acid while the σ_p of aromatic aldehydes is derived from pK_a of benzoic acid. Therefore, the relationships between σ_p and M for aromatic aldehydes and aliphatic saturated aldehydes are obtained in eqs 7 and 8, respectively.

$$M = 0.788 - 0.882\sigma_p \qquad (7)$$

where $n = 8$, $r^2 = 0.890$, SE = 0.129, $F = 48.417$, and $P = 0.000$ and

$$M = 0.0015 - 4.876\sigma_p \qquad (8)$$

where $n = 6$, $r^2 = 0.922$, SE = 0.099, $F = 47.081$, and $P = 0.002$. The significant correlation for both eqs 7 ($r^2 = 0.890$) and 8 ($r^2 = 0.922$) indicates success in developing QSAR models to describe the relationship between the joint effects and the chemical structures of aldehydes.

Relationships between the Joint Effects and the Chemical Structures of Cyanogenic Toxicants. As shown in Figure 3, during the formation of the carbanion intermediate, cyanogenic toxicants act essentially via their physiologically generative product (CN^-). This indicates that for cyanogenic toxicants, although the joint effects between either of them and the aldehyde are different, they contribute the same (CN^-) to the formation of the carbanion intermediate. The different contribution of cyanogenic toxicants to the joint effects therefore can be attributed to their different generation of CN^-. Comparison of this generation will reveal the relationships between the joint effects and the chemical structures of cyanogenic toxicants.

According to the findings of Lipnick (*12*), for the cyanogenic toxicants listed in Table 3, CN^- is generated via monooxygenase activation. For example, the detailed generation of CN^- from acrylonitrile and malononitrile is shown in Figure 2. It can be seen from Figure 2 that being attacked by the monooxygenase enzyme, some carbon atoms (C*) in the carbochain of cyanogenic toxicant take on significant radical character, thereby readily accepting the offense from the −OH group, thus

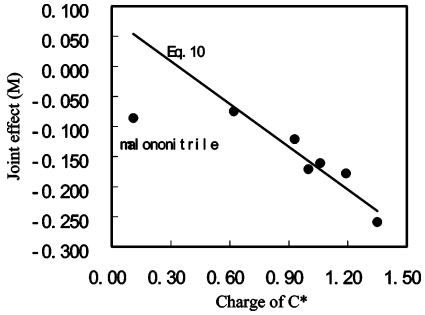


Figure 8. Relationship between the joint effect (*M*) and the charge of C* for individual cyanogenic toxicants.

leading to a cyanohydrin type intermediate, which readily generates CN^- upon hydrolysis. On the basis of this procedure, one can conclud that the generation of CN^- depends on the positive charge (C*) because of the anionic nature of the −OH group. In other words, there is a correlation between C* and the generation of CN^-: the more positive the charge of C*, the greater attraction for the negative charge of the −OH group, the easier the formation of the cyanohydrin type intermediate, and the easier the generation of CN^- is. According to this correlation, quantification of the positive charge of C* would allow the generation of CN^- to be evaluated; therefore, the relationships between the joint effects and the chemical structures of cyanogenic toxicants could be revealed.

Using the MOPAC (version 6.0) program (*18*), the charge of C* in the various cyanogentic toxicants was calculated and the results are listed in Table 3. As shown in Table 3, the positive charge of C* in acetonitrile (−0.259) is less than that in acrylonitrile (−0.161). Consistent with this less positive charge of C*, the slower generation of CN^- was observed by He (*19*). When studying the toxicity for acrylonitrile and acetonitride to mice, he observed that the CN^- peak released by acrylonitrile in the blood of mice appeared 2 h after administrated, whereas the peak by acetonitrile appeared after 8 h. This result provides further support for use of the positive charge of C* for estimating the generation of CN^- from the cyanogenic toxicants. By using this parameter (C*), the relationships between the joint effects and the chemical structures of cyanogenic toxicants are shown in Figure 8 and eq 9

$$M = -0.0353 - 0.128C^* \quad (9)$$

where $n = 7$, $r^2 = 0.711$, SE = 0.037, $F = 12.322$, and $P = 0.017$. However, only a poor correlation coefficient ($r^2 = 0.711$) is obtained in eq 9. As shown in Figure 8, this is due to the outlier of malononitrile; there are double −CN groups in malononitrile and either of them can lose a CN^- (Figure 2b), whereas there is only one −CN group in the other cyanogenic toxicants in this study. Deletion of malononitrile from eq 9 leads to the following equation

$$M = 0.0824 - 0.237C^* \quad (10)$$

where $n = 6$, $r^2 = 0.913$, SE = 0.020, $F = 41.861$, and $P = 0.003$. The significant correlation ($r^2 = 0.913$) in eq 10 indicates success in developing a QSAR to describe the relationship between the joint effects and the chemical structures of cyanogenic toxicant. However, before these novel QSAR approaches can be used generally, a wider range of chemicals has to be tested to further validate the approaches' capability because the QSARs in this study are only derived from the mixtures containing aldehydes and cyanogenic toxicants.

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