Hydrolytic and Toxicological Properties of Ethyl α-Cyanobenzaldoxime Phosphoramidates

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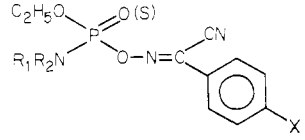
A series of ethyl alkylphosphoramidate esters of α-cyanobenzaldoxime and ring-substituted α-cyanobenzaldoximes were synthesized and examined for alkaline hydrolysis, anticholinesterase activity, and toxicity to houseflies and mice. The compounds showed variable toxicity to houseflies and mice and were effective inhibitors of acetylcholinesterase. Satisfactory correlation was obtained between alkaline hydrolysis rate constants and anticholinesterase activity with Taft's σ^* and E_s values. Toxicity to houseflies was related to anticholinesterase activity and Hansch's π constants. Several of the compounds were of low toxicity to the white mouse but were highly effective against the housefly.

Previous studies in this laboratory concerning the selective toxicity of phoxim (*O,O*-diethyl *O*-α-cyanobenzaldoxime phosphorothioate), the corresponding oxon and related analogues revealed the high safety to mammals and high insecticidal activity of these compounds (Vinopal and Fukuto, 1971). The favorable properties of selectivity were explained on the basis of differences in rates and routes of detoxication in the insect and mammal, coupled with differences in sensitivity of insect and mammalian acetylcholinesterase (AChE) to inhibition by the oxon.

Because of the desired order of selectivity demonstrated by phoxim and analogues and the limited amount of information concerning the relationship of chemical structure and biological activity of phosphoramidate esters, it was of interest to examine a series of phosphoramidates containing the α-cyanobenzaldoxime leaving group which is present in phoxim. This report is concerned with the synthesis and toxicological evaluation of phosphoramidates of the general structure shown below where R_1 and R_2 are hydrogen or small alkyl groups and X is H, Cl, NO_2, Me, or MeO groups.



MATERIALS AND METHODS

Nitriles. Phenyl-, *p*-chlorophenyl-, and *p*-methylphenylacetonitriles were obtained from Aldrich Chemical Co. *p*-Nitrophenylacetonitrile was prepared by reacting phenylacetonitrile with fuming nitric acid (Schonne et al., 1953). The product was recrystallized from ethanol, mp 111–113 °C. *p*-Methoxyphenylacetonitrile was prepared as previously described (Rorig et al., 1963).

Oximes. α-Cyanobenzaldoxime and the substituted analogues were prepared by nitrosylation of the phenylacetonitriles according to Perrot (1934). The products were recrystallized from water; melting points: α-cyanobenzaldoxime, 124–125 °C; *p*-Cl, 109–111 °C; *p*-NO_2, 159–161 °C. *p*-Methoxy- and *p*-methyl-α-cyanobenzaldoxime could not be crystallized, and the oily products were converted to the solid sodium salts which were characterized by NMR.

Phosphoramidates. α-Cyanobenzaldoxime ethyl phosphorochloridate and ring-substituted analogues were prepared by reacting the sodium salt of the oxime with 1 equiv of ethyl phosphorodichloridate in toluene. The reaction mixture was then treated with ammonia or the desired amine to obtain the α-cyanobenzaldoxime ethyl phosphoramidate. Typically, to 2.2 g (0.013 mol) of ethyl phosphorodichloridate in 100 mL of dry toluene was added slowly 2.1 g (0.015 mol) of α-cyanobenzaldoxime sodium salt. The mixture was warmed to 40–50 °C for 1 h with stirring and the precipitated sodium chloride was removed by filtration. Dry ether (100 mL) was added to the filtrate and methylamine was bubbled into the mixture until precipitation of methylamine hydrochloride ceased. The mixture was filtered and the filtrate was washed with 0.5% aqueous sodium hydroxide and water, dried, and concentrated to give the crude product. Recrystallization from ether–hexane yielded 1.2 g of α-cyanobenzaldoxime ethyl *N*-methylphosphoramidate (**2**), mp 105–106 °C.

α-Cyanobenzaldoxime *O*-ethyl *N*-methylphosphoramidothioate (**14**) was prepared by reacting *O*-ethyl *N*-methylphosphoramidochloridothioate (Mel'nikov et al., 1967) with the sodium salt of α-cyanobenzaldoxime. The product was purified as described above for the phosphoramidates. Elemental analyses and physical properties for all final products are given in Table I. Support for the indicated structures also was obtained by NMR using a Varian T-60 spectrometer. Microanalyses were by C. F. Geiger, Ontario, Calif.

Toxicological Procedures. Bimolecular rate constants (k_i) for the inhibition of housefly-head acetylcholinesterase (HFAChE) and bovine erythrocyte acetylcholinesterase (BAChE) (Sigma Chemical Co.) were determined at 30 °C as previously described (Fukuto and Metcalf, 1956) using acetylthiocholine as the substrate (Ellman et al., 1961). It was necessary to add Triton X-100 (1%) to the HFAChE homogenate to avoid curvilinear pseudo-first-order plots of the inhibition reaction, as was previously observed with another series of phosphoramidothioates (Sanborn and Fukuto, 1972).

Toxicity to the susceptible NAIDM strain of houseflies (*Musca domestica*) was determined according to March and Metcalf (1949). Mammalian toxicity was determined orally on Swiss white mice using propylene glycol as carrier according to usual methods (Hollingworth et al., 1967).

Hydrolysis. Second-order constants for the alkaline hydrolysis of the oxime phosphoramidates (**1–13**) were determined in 0.005 M sodium hydroxide at 30 °C by following the formation of the oxime anion at 324 nm for compounds **1–7**, 330 nm for compounds **8–10**, 360 nm for **11** and 335 nm for 13. The concentration of the phosphoramidates was approximately 5×10^{-5} M. Owing to

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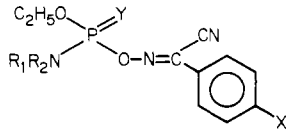
Table I. Physical Constants of Phosphoramidates of Indicated Structure



no.	R_1	R_2	X	Y	mp, °C	analysis: theory	analysis: found
1	H	H	H	O	150-151	C, 47.44 H, 4.78	C, 47.68 H, 4.53
2	H	Me	H	O	105-106	C, 49.44 H, 5.28	C, 49.21 H, 5.82
3	H	Et	H	O	115-116	C, 51.25 H, 5.73	C, 50.89 H, 5.68
4	H	*n*-Pr	H	O	102-103	C, 52.88 H, 6.14	C, 52.92 H, 6.67
5	H	*i*-Pr	H	O	100-101	C, 52.88 H, 6.14	C, 53.12 H, 6.28
6	H	*t*-Bu	H	O	95-96	C, 54.36 H, 6.52	C, 55.03 H, 6.46
7	Me	Me	H	O	oil[a]	C, 51.25 H, 5.73	C, 51.58 H, 6.04
8	H	Me	Cl	O	116-117	C, 43.78 H, 4.34	C, 43.96 H, 4.74
9	H	Et	Cl	O	104-106	C, 45.60 H, 4.79	C, 45.84 H, 4.87
10	H	*n*-Pr	Cl	O	86-87	C, 47.35 H, 5.20	C, 46.87 H, 4.73
11	H	Et	NO_2	O	125-126	C, 44.17 H, 4.63	C, 43.80 H, 4.80
12	H	Et	MeO	O	84-85	C, 50.16 H, 5.83	C, 50.10 H, 5.43
13	H	Et	Me	O	83-84	C, 52.88 H, 6.15	C, 53.06 H, 5.95
14	H	Me	H	S	55-57	C, 46.63 H, 4.98	C, 47.03 H, 5.02

[a] $n^{25}_D = 1.5195$.

Table II. Toxicological and Hydrolytic Properties of Substituted Ethyl α-Cyanobenzaldoxime Phosphoramidates

no.	compound[a] R_1	R_2	X	Y	k_i, M^{-1} min^{-1} HFAChE	k_i, M^{-1} min^{-1} BAChE	LD_{50}, mg/kg housefly	LD_{50}, mg/kg mouse	$k_b \times 10^{-2}$, M^{-1} min^{-1}	log *P*
1	H	H	H	O	2.0×10^8	1.7×10^8	>500		3.8×10^5	0.72
2	H	Me	H	O	2.8×10^7	4.5×10^6	11.7	10	2.15	0.96
3	H	Et	H	O	9.5×10^6	9.6×10^5	17.5	75	1.41	1.46
4	H	*n*-Pr	H	O	7.5×10^6	5.0×10^5	22.5	>500	1.35	1.96
5	H	*i*-Pr	H	O	2.1×10^5	1.6×10^5	45	150-200	0.261	1.76
6	H	*t*-Bu	H	O	8.8×10^4	2.0×10^4	>500		0[b]	2.14
7	Me	Me	H	O	1.3×10^5	1.2×10^4	90	<50	0[b]	3.29
8	H	Me	Cl	O	1.3×10^7	1.2×10^6	3.9	50-75	0.989	2.50
9	H	Et	Cl	O	3.1×10^6	3.5×10^5	3.6	>500	0.605	3.00
10	H	*n*-Pr	Cl	O	8.4×10^5	1.3×10^5	8.3	>500	0.636	3.50
11	H	Et	NO_2	O	7.1×10^6	8.2×10^5	>500 (7.0)[c]		1.73	1.76
12	H	Et	MeO	O	2.6×10^6	8.4×10^4	>500 (7.3)[c]			2.39
13	H	Et	Me	O	3.0×10^6	1.7×10^5	>500 (11.5)[c]		0.818	1.46
14	H	Me	H	S			>300			

[a] See Table I for structure. [b] No detectable evidence for hydrolysis after 24 h in 0.05 M NaOH. [c] With piperonyl butoxide 5:1 (w/w).

its extreme sensitivity to base hydrolysis, k_b for compound 1 was determined in pH 7.6 phosphate buffer. Spectra of the various phosphoramidates and hydrolysis products were examined prior to the kinetic studies to show that overlap in relevant absorption peaks were not present.

Partition coefficients (*P*) between octanol and water for the phosphoramidates 1, 7, 8, 11, and 12 were obtained experimentally using a Beckman Model 25 UV-spectrophotometer to determine the amount of compound in each phase. Other values of *P* were obtained by use of the additivity principle (Hansch, 1971). The free-energy parameters σ^* and E_s were from Taft (1956) and π was calculated from the values of the partition coefficients (Fujita et al., 1964).

RESULTS

Hydrolysis. Rates of alkaline hydrolysis were determined spectrophotometrically by estimating the amount of oxime formed under pseudo-first-order conditions of excess sodium hydroxide. Second-order hydrolysis constants (k_b) for the various benzaldoxime phosphoramidates are presented in Table II.

For the phosphoramidates without ring substituents, i.e., compounds 1–7, rate constants were determined for the

first five compounds. The *tert*-butyl- (**6**) and dimethylamido (**7**) analogues were very stable, and no evidence of hydrolysis was obtained for either compound in 0.05 M sodium hydroxide after 24 h. Regression analysis of the data with Taft's (1956) σ^* and E_s constants for compounds **1–5** resulted in very good correlation with each parameter according to the following equations:

$$\log k_b = 9.10\sigma^* - 1.48 \quad (1)$$

$$r = 0.989 \quad n = 5 \quad F = 138.8$$

$$\log k_b = 3.61E_s - 1.60 \quad (2)$$

$$r = 0.981 \quad n = 5 \quad F = 78.0$$

where n is the number of compounds, r is the correlation coefficient, and the F value is the statistical measure of the significance of the correlation.

Unfortunately, there was a high degree of collinearity between σ^* and E_s for the amido substituents in compounds **1–5** ($r = 0.99$) and it was difficult to establish which of the two parameters was more important in correlating the hydrolysis data. Inclusion of both σ^* and E_s in the regression analysis gave:

$$\log k_b = 8.84\sigma^* + 0.10E_s - 1.49 \quad (3)$$

$$r = 0.989 \quad n = 5 \quad F = 46.3$$

The coefficients in the equation indicate a greater dependence on σ^* than E_s but steric effects undoubtedly become more important as the size of the amido substituent becomes larger. This is illustrated by data obtained with **6** (NH-*t*-Bu) which showed no evidence of hydrolysis after 24 h in 0.05 M sodium hydroxide. From eq 1, using $\sigma^* = 0.19$ for *t*-Bu, the calculated k_b for **6** was 1.78 M^{-1} min^{-1}. Based on this rate constant, the half-life ($t_{1/2}$) of **6** in 0.05 M sodium hydroxide was calculated to be 7.8 min, a value which is at least two orders of magnitude greater than the actual half-life.

Although only a few ring-substituted benzaldoxime phosphoramidates were prepared and examined for hydrolysis (**8–13**), analysis of the data showed no trend between structure and hydrolysis rates. This is evident from the k_b values for **3**, **9**, **11**, and **13**, i.e., analogues which are identical except for the ring substituent. Owing to overlaps in relevant absorption peaks, k_b for **12** (*p*-OCH_3) could not be determined. Since alkaline hydrolysis occurs by cleavage of the phosphorus–oxime oxygen bond, ring substituents which are electron-withdrawing should increase hydrolysis rates. While the *p*-nitro derivative (**11**), with k_b = 173.9 M^{-1} min^{-1}, was most susceptible to hydrolysis, the *p*-chloro analogue (**9**) was least susceptible. In fact, **9**, with the electron-withdrawing chlorine substituent, was distinctly more stable to alkaline hydrolysis than the unsubstituted phosphoramidate (**3**) and compound **13**, containing the electron-donating *p*-methyl group.

Cholinesterase Inhibition. Bimolecular constants (k_i) for the inhibition of acetylcholinesterase show that the various phosphoramidates were potent anticholinesterases and, in general, were stronger inhibitors of HFAChE than BAChE. Evidence for greater anticholinesterase activity toward HFAChE is shown graphically in Figure 1 which gives the relation between log k_i and $-\log k_b$ for the *N*-alkylphosphoramidates for which k_b and k_i values were obtained. Overall, the plot reveals a linear relationship between the logarithms of the rate constants for inhibition and hydrolysis, and anticholinesterase activity against HFAChE is about tenfold greater than against BAChE. Progressive increases in the size of the amido alkyl substituent resulted in a decrease in anticholinesterase activity (compare compounds **1–6** and **8–10**) against both enzymes.

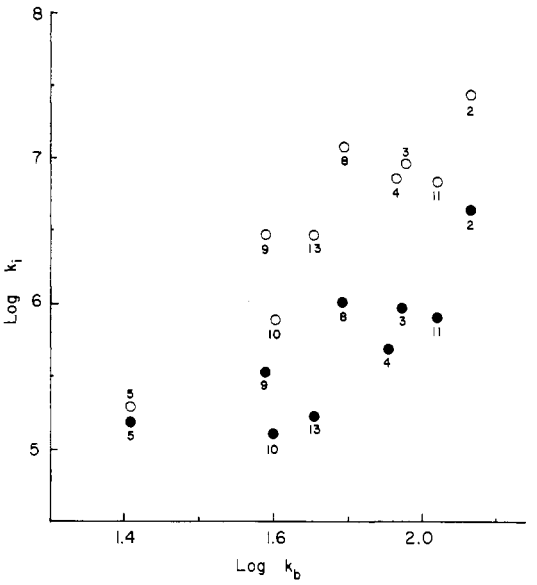


Figure 1. Relation between log k_b for alkaline hydrolysis and log k_i for the inhibition of HFAChE (○) and BAChE (●).

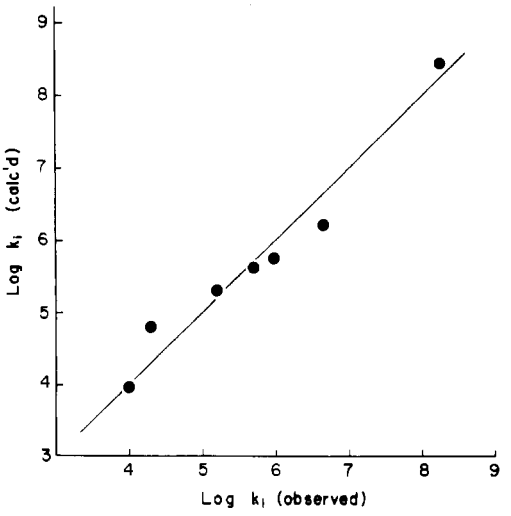


Figure 2. Correlation between observed log k_i and log k_i calculated according to eq 4 for the inhibition of BAChE.

Regression analysis of the inhibition data for compounds **1–7** against BAChE with σ^*, E_s, and π resulted in eq 4 which gave best fit to the data:

$$\log k_i = 2.34E_s + 0.8\sigma^* + 4.10 \quad (4)$$

$$r = 0.990 \quad n = 7 \quad F = 98.2$$

In spite of their collinearity, the inclusion of σ^* and E_s together in the equation was statistically significant at the 0.05 level of probability, providing evidence that anticholinesterase activity was governed mainly by polar and steric effects associated with the amido moiety. A similar equation was obtained previously in rationalizing the anticholinesterase activity of a series of methyl 2,4,5-trichlorophenyl phosphoramidates (Fukuto et al., 1963; Hansch and Deutsch, 1966). The relationship between observed k_i, σ^*, and E_s is shown graphically in Figure 2.

Analysis of the data for inhibition of HFAChE (**1–7**) resulted in eq 5 which gave best fit to the data:

$$\log k_i = 1.33E_s + 5.23 \quad (5)$$

$$r = 0.923 \quad n = 7 \quad F = 28.8$$

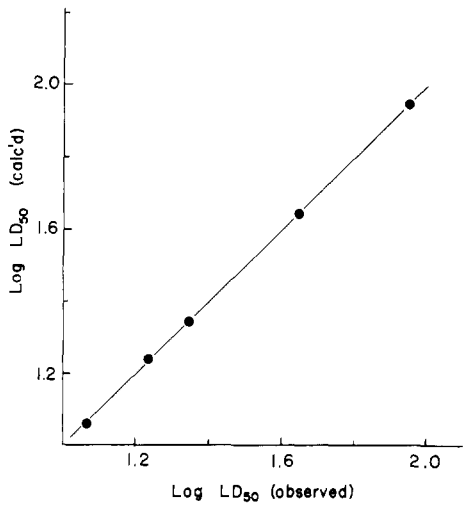


Figure 3. Correlation between observed log LD_{50} to houseflies and log LD_{50} calculated according to eq 6.

Inclusion of σ^* in the regression analysis surprisingly had no effect on the correlation, indicating the absence of polar influence by the amido moieties on HFAChE inhibition. Owing to the presence of σ^* in the equation involving BAChE inhibition and the linear relationship observed between log k_i and log k_b for HFAChE inhibition, the absence of σ^* in eq 5 is difficult to explain. However, as mentioned earlier, σ^* and E_s are collinear, and it is possible that only one parameter is accounting for both steric and polar effects. Use of σ^* alone in the regression resulted in poorer correlation. Further, introduction of π in eq 4 and 5 had no effect on the correlation.

Analysis of the inhibition data by the ring substituted phosphoramidates (**3**, **9**, **11**, **12**, **13**) against HFAChE and BAChE resulted in poor correlation (r = 0.873 and 0.814, respectively) with σ and π. The results were not statistically significant at the 0.05 level of probability.

Toxicity. The phosphoramidates in Table II showed variable but interesting toxicity to houseflies and mice. For the compounds without substituents in the phenyl ring (**1**–**7**), increase in size of the amido alkyl substituent resulted in a gradual decrease in housefly toxicity. Despite its high anticholinesterase activity, the unsubstituted amido analogue **1** was nontoxic to flies and this was probably attributable to its high hydrolytic instability. Compared to flies, a drastic decrease in mouse toxicity with increasing alkyl size was observed (compare **2**, **3**, and **4**).

According to Hansch (Hansch et al., 1965), the amount of externally applied chemical arriving at a target site to produce a biological response should be related to the octanol–water partition parameter π. Except for differences in rates of metabolism, the toxicity of direct inhibitors of AChE, such as the phosphoramidates in this study, should be a function of π and their intrinsic anticholinesterase activity. In agreement with this type of model, an almost perfect linear relationship was obtained in correlating housefly toxicity with π and k_i values for HFAChE:

$$\log LD_{50} = 0.16\pi - 0.21 \log k_i + 2.56 \qquad (6)$$

$$r = 0.999 \quad n = 5 \quad F = 7555$$

Compounds included in eq 6 are those for which finite housefly LD_{50} values were obtained, i.e., **2**, **3**, **4**, **5**, and **7**. The results are presented graphically in Figure 3 which shows the relation between observed log LD_{50} and calculated log LD_{50}. Although the high degree of correlation may be purely fortuitous owing to the small number of compounds, nevertheless, the equation points out the equal importance of hydrophobic effects and anticholinesterase activity in determining toxicity.

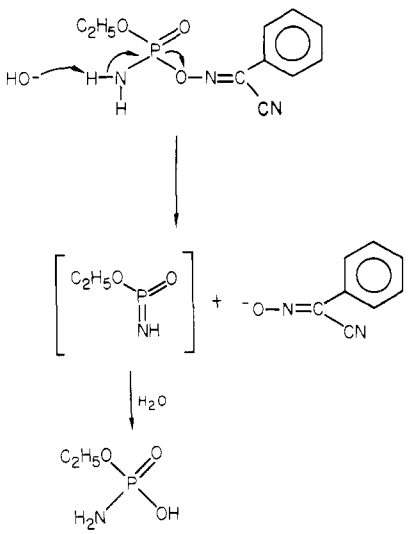
Placement of substituents on the phenyl ring had a profound effect on housefly toxicity. For example, introduction of a chlorine substituent in the para position increased housefly toxicity three- to five-fold (compare **2**, **3**, **4** with **8**, **9**, **10**) but substitution with nitro, methoxy, and methyl groups resulted in compounds which were nontoxic at 500 μg/g. The latter three compounds (**11**, **12**, **13**), however, were strongly synergized by cotreatment with 5:1 parts of piperonyl butoxide. In fact, the synergism of more than seventy-fold observed for **11** and **12** with piperonyl butoxide is unusual for an organophosphorus ester and is even greater than the level of synergism observed for carbamate esters. Because of their high anticholinesterase activity the low toxicity of **11**, **12**, and **13** when applied alone to houseflies was surprising. These compounds evidently are highly susceptible to oxidative detoxication, as indicated by their strong response to piperonyl butoxide.

For reasons which are not obvious, the *p*-chloro substituted phosphoramidates were less toxic to mice than the corresponding unsubstituted analogues (compare **2**, **3**, **4** with **8**, **9**, **10**). However, the same trend of decreasing toxicity with increase in size of the amido substituent also was observed. Compound **9**, ethyl α-cyano-*p*-chlorobenzaldoxime ethylphosphoramidate, with LD_{50} values of 3.6 μg/g to houseflies and >500 mg/kg to mice, showed favorable properties of selectivity and is worthy of further examination as a useful insecticide.

The single phosphoramidothioate analogue examined (**14**) was nontoxic to flies at 300 μg/g, indicating poor in vivo metabolic activation to the oxon (**2**) since the latter is a strong anticholinesterase and of moderate housefly toxicity.

DISCUSSION

Previous studies in this laboratory (Sanborn and Fukuto, 1972); Quistad et al., 1970; Fahmy et al., 1972; Fukuto et al., 1963) have revealed the profound effect of small changes in structure on the reactivity and toxicological properties of phosphoramidothioates and phosphoramidates. This was also the situation for the substituted α-cyanobenzaldoxime phosphoramidates examined in this study and in some cases small changes in structure produced even greater effects than previously observed with other phosphoramidate esters. For example, replacement of the amido proton in **1** with a methyl group to give **2** resulted in greater than 10^5-fold decrease in the second-order rate constant for base-catalyzed hydrolysis. In comparison, substitution of one of the protons in *O*-ethyl *S*-*p*-chlorophenyl phosphoramidothioate with a methyl gave only a 50-fold reduction in rate constant (Sanborn and Fukuto, 1972). The same substitution in *O*,*S*-dimethyl phosphoramidothioate resulted in 110-fold decrease in hydrolysis rate constant (Fahmy et al., 1972). The 10^5-fold difference in k_b between **1** and **2** and the exceedingly high hydrolytic stability of the *N*,*N*-dimethyl analogue (**7**) again raises the question of the formation of a metaphosphorimidate intermediate in the hydrolytic process (Traylor and Westheimer, 1965; Fahmy et al., 1972). The *N*,*N*-dimethyl analogue **7** showed no evidence of hydrolysis after standing in 0.05 M NaOH for 24 h. Based on this observation, **7** is at least 10^4-fold more stable to alkaline hydrolysis than the monomethyl analogue **2** and the difference is probably much greater. The large differences in hydrolysis rates between **1**, **2**, and **7** is difficult to explain in terms of a single mechanism involving displacement of the benzaldoxime moiety by hydroxide ion attack on the



phosphorus center. Hydrolysis of **7** must take place by attack of hydroxide on the phosphorus but owing to their much larger rate constants, hydrolysis of **1** and **2** probably occurs by attack of hydroxide on the amido proton.

The α-cyanobenzaldoxime phosphoramidates were surprisingly strong inhibitors of AChE and activity appeared to be a function of both polar and steric effects. The relatively high anticholinesterase activity of the *N,N*-dimethyl analogue **7** was unexpected because of its low reactivity toward hydroxide ion.

The large differences in housefly and mouse toxicity observed for compounds **4**, **9**, and **10** cannot be rationalized in terms of differences in inhibition between housefly-head and mammalian AChE since the differences in enzyme inhibition were more or less the same with all compounds. The toxicity differences probably are attributable to differences in rates and routes of detoxication in houseflies and mice, as suggested for the related diethyl ester analogue phoxim (Vinopal and Fukuto, 1971).

The interesting toxicological properties of the α-cyanobenzaldoxime phosphoramidates justify further examination of compounds of this type as potential insecticides.

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Toxicity, Accumulation, and Depletion of Hexachlorobenzene in Laying Chickens

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Laying pullets were given seven consecutive daily oral doses of hexachlorobenzene (HCB) ranging from 1 to 100 mg/kg. At higher doses, HCB delayed the onset of full egg production but also appeared to offer protection against development of hemorrhagic fatty liver. Relative accumulation of HCB in fat was inversely related to dose, but elimination from fat was constant for residue concentrations from 3–800 ppm. Elimination half-time from fat was estimated at 24–27 days and more than half of the elimination was due to parent compound excreted in egg yolks. Other tissues generally paralleled fat, but skin became an increasingly significant reservoir and liver concentrations remained rather constant.

Hexachlorobenzene (HCB) is a chlorinated hydrocarbon dispersed in the environment and in animal feeds (Burns and Miller, 1975; Yang et al., 1976). HCB has a low acute toxicity, but causes a variety of ill effects during and after prolonged exposure (Booth and McDowell, 1975).

Feeding studies in poultry indicate that concentrations up to 100 ppm HCB do not produce notable ill effects, but residue accumulation is quite marked (Avrahami and Steele, 1972a,b; Reed et al., 1977a,b). The following study was undertaken using a short-term, more intensive dosing

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