Inhibition of Microsomal Drug Metabolism by Anticholinesterase Insecticides

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With the increasing awareness of the problems associated with environmental pollutants there is a growing reluctance to continue use of the so-called "hard pesticides" or organochlorines. As a result the use of organophosphate insecticides as substitutes for organochlorine compounds has increased. Indeed, organophosphate insecticides have now become the most widely used agricultural pesticides. Various aspects of organophosphate toxicity have been discussed from the viewpoint of health hazards (DAVIES et al. 1967, DUBOIS 1967, FOUTS 1970).

It is well known that organophosphates and carbamates serve as substrates for the hepatic microsomal enzymes (O'BRIEN 1967). Many organophosphates require microsomal oxidation to the toxic form, hence altered microsomal activity due to enzyme induction by other chemicals could result in diminished or enhanced toxicity depending upon whether oxidative degradation or activation predominates (O'BRIEN 1967). The effect of a known inhibitor of drug metabolism such as SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl) and inducers such as DDT, 3,4-benzpyrene and phenobarbital on the in vitro metabolism of parathion has been reported (VILLENEUVE 1970). It was observed that SKF 525-A had no effect on the toxicity of parathion while DDT, 3,4-benzpyrene and phenobarbital treatment decreased parathion toxicity three-fold. These results demonstrate that interactions at the microsomal level are important determinants of organophosphate actions.

Inasmuch as organophosphates are known to serve as substrates for hepatic microsomal enzymes it is also possible that they may act as inhibitors of this system. Studies designed to test this hypothesis are presented herein. Specifically, results on the inhibitory effects of anticholinesterase insecticides on hepatic microsomal drug metabolism are reported.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 100-150 g were used as experimental animals. Hepatic microsomal enzymes were isolated as described previously (ANDERS 1968). Incubation mixtures contained varying quantities of substrate and inhibitor (as indicated in the table), 15 µmoles magnesium chloride, 50 µmoles phosphate buffer (pH 7.4), a NADPH-generating system consisting of 10 µmoles DL-isocitrate, 1 µmole NADP and 1 enzyme unit of isocitrate dehydrogenase and 0.5 ml of microsomal enzyme (12 mg protein/ml), in a

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total volume of 3.0 ml. Incubations were carried out at 37° with shaking in an atmosphere of air. The enzyme preparation was added after preincubation period of 10 min to ensure temperature constancy. Incubation times were 10 min in the case of ethylmorphine and aniline and 6 min for the metabolism of p-nitroanisole; during these time periods product formation was linear with time. Inhibitors were added to the incubation mixtures in 10 µl of methanol. As most solvents inhibit aniline hydroxylation, the inhibitor was added to the flask and the solvent evaporated before other components of the reaction mixture were added. When formaldehyde was the product to be measured, 3 µmoles of neutralized semicarbazide HCl were added to the reaction mixture; formaldehyde was analyzed according to the method of NASH (1953). p-Aminophenol produced by the hydroxylation of aniline was measured according to the method of KATO and GILLETTE (1960) and p-nitrophenol formed by the 0-demethylation of p-nitroanisole as described by NETTER (1960).

Kinetic constants for the enzymatic reactions were determined by computer according to the method of CLELAND (1967). The inhibition constant (K_i) was calculated from the slope ratio of the inhibited and uninhibited curves as described by WEBB (1963). Spectral binding studies were conducted as described by SCHENKMAN et al. (1967). The cuvettes contained 1 mg/ml of microsomal protein in 0.1 M phosphate buffer, pH 7.4; 1 mM organophosphate was added to the sample cuvette in acetone solution. This solvent produces no difference spectrum with rat hepatic microsomal fractions (unpublished observations).

RESULTS AND DISCUSSION

Results of *in vitro* inhibition studies by various anticholinesterase insecticides are presented in Table 1. Data obtained with a known inhibitor of hepatic microsomal drug meta-

TABLE 1

In Vitro Inhibition of Microsomal Drug Metabolism by Anticholinesterase Insecticides

	I_{50} (M)		
	Substrate		
Inhibitor	Ethylmorphine[a]	p-Nitroanisole[b]	Aniline[c]
Parathion	$3.8\pm0.4 \times 10^{-6}$(5)	---[d]	$2.9\pm1.7 \times 10^{-5}$(4)
Malathion	$1.3\pm0.3 \times 10^{-4}$(5)	$1.1\pm0.4 \times 10^{-4}$(3)	$3.3\pm0.9 \times 10^{-5}$(4)
Carbaryl	$2.1\pm0.6 \times 10^{-3}$(4)	$6.7\pm2.1 \times 10^{-4}$(3)	$8.4\pm5.5 \times 10^{-6}$(8)
SKF 525-A	$0.8\pm0.2 \times 10^{-5}$(3)	$0.3\pm0.1 \times 10^{-5}$(3)	$2.0\pm0.4 \times 10^{-3}$(3)

[a] Substrate concentration: 8.0×10^{-4} M.
[b] Substrate concentration: 4.0×10^{-4} M.
[c] Substrate concentration: 1.0×10^{-3} M.
[d] Inhibitor interferes with measurement of p-nitrophenol derived from p-nitroanisole.

bolism, SKF 525-A, is included for comparison. With ethylmorphine as the substrate, parathion was approximately equipotent with SKF 525-A as an inhibitor. In the case of p-nitroanisole as the substrate, both malathion and carbaryl were less potent than SKF 525-A. Although carbaryl proved to be the most potent inhibitor of aniline hydroxylation all of the anticholinesterases tested were approximately one hundred-fold more potent than SKF 525-A as inhibitors of this reaction. The reasons for the sensitivity of the aromatic hydroxylase to these compounds are not clear. While the detailed mechanism by which anticholinesterase compounds inhibit microsomal drug metabolism is not known, it is reasonable to speculate that these compounds serve as alternative substrate inhibitors. This contention is based on the knowledge that the sulfur form of organophosphates undergoes microsomal oxidation to yield the oxygenated organophosphates which are potent inhibitors of cholinesterase. Presumably both the anticholinesterase and the substrates tested compete for the same microsomal enzyme system and thereby produce the observed inhibition.

The oxygen analogues of parathion, paraoxon, and malathion, malaoxon, have been studied as inhibitors of drug metabolism. The I_{50} for the inhibition of ethylmorphine N-demethylation by parathion and paraoxon was 3.8×10^{-6} (Table 1) and $>10^{-3}$ M, respectively; similarly, the I_{50} for malathion and malaoxon was 1.3×10^{-4} (Table 1) and 1×10^{-3} M, respectively. The finding that paraoxon and malaoxon are very poor inhibitors of ethylmorphine metabolism agrees with other reports (WELCH et al. 1967, CONNEY et al. 1967) demonstrating that oxygenated organophosphates are weak inhibitors of microsomal testosterone metabolism. The lack of inhibitory effectiveness of these compounds is probably attributable to their failure to serve as substrates for the microsomal mixed function oxidase system. Indeed, this observation supports the contention that organophosphates inhibit microsomal enzymes by serving as alternative substrates. These findings agree with those of RUBIN et al. (1964) who reported that of a number of compounds tested only those undergoing metabolism would serve as inhibitors of microsomal oxidations.

The kinetics of the inhibition of ethylmorphine N-demethylation and aniline hydroxylation by parathion have also been studied. The inhibition constant (K_i) of parathion for ethylmorphine and aniline metabolism was 2.4×0.3 μM ($n = 20$) and 4.6 ± 1.4 μM ($n = 4$), respectively. According to the classification of WEBB (1963), the type of inhibition produced by parathion was mixed in the case of ethylmorphine and uncompetitive when aniline served as the substrate. While competitive inhibition is frequently observed with the microsomal system (ANDERS and MANNERING 1966), SASAME and GILLETTE (1970) have demonstrated that the type of inhibition obtained is a function of several variables including the nature of the binding spectrum, concentration of inhibitor and species. It should also be noted that failure to obtain competitive kinetics does not necessarily rule out an alternative substrate mechanism of inhibition but does suggest that the interaction between the substrate,

inhibitor and enzyme is complex.

As can be seen in Fig. 1 and 2, parathion and malathion showed Type I binding spectra with oxidized hepatic microsomal fractions (SCHENKMAN et al. 1967); the spectral binding constant (K_s) were 1.2 x 10^{-4} M and 0.43 x 10^{-4} M, respectively. Both paraoxon and malaoxon produced atypical binding spectra (Fig. 1 and 2). The failure of the oxygenated organophosphates to produce

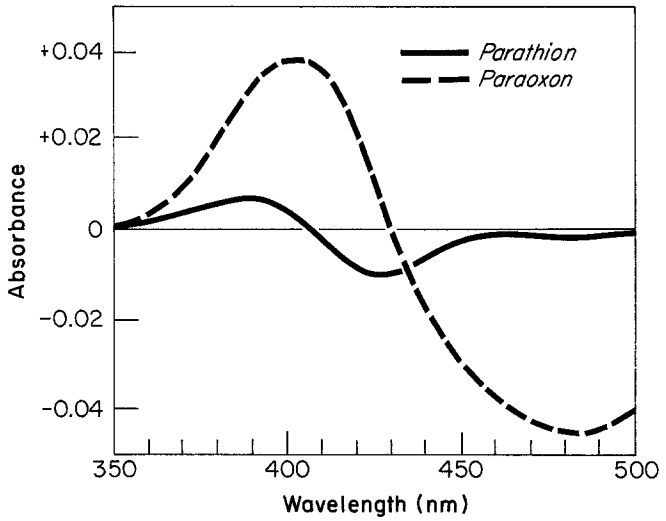

Fig. 1. Parathion and paraoxon difference spectra with oxidized rat hepatic microsomal fractions.

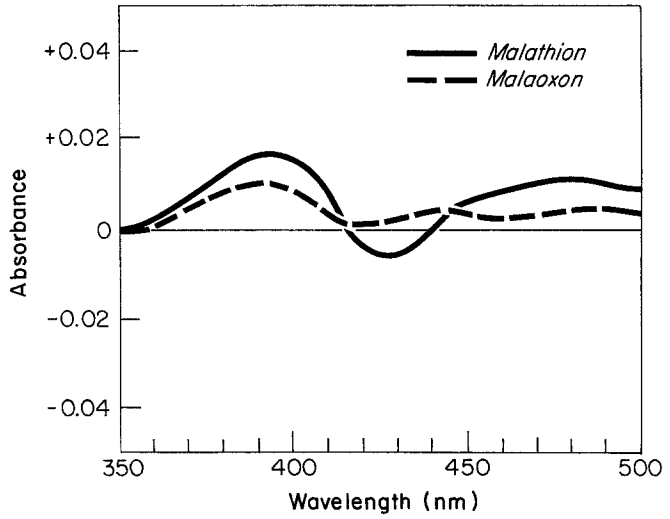

Fig. 2. Malathion and malaoxon difference spectra with oxidized rat hepatic microsomal fractions.

binding spectra again suggests that these compounds do not interact in the usual manner with microsomal hemoproteins.

The inhibition of hepatic microsomal drug metabolism in the intact animal by anticholinesterases has been studied using hexobarbital sleeping time and zoxazolamine paralysis time as indicators of drug metabolism. As can be seen in Table 2, neither malathion nor dimethylsulfoxide (DMSO), the vehicle employed, produced detectable inhibition of either hexobarbital or zoxazolamine metabolism; in contrast, SKF 525-A was an effective inhibitor of the in vivo metabolism of both compounds. The dose of DMSO

TABLE 2

Effect of Malathion on the In Vivo Metabolism of Hexobarbital and Zoxazolamine

Inhibitor	Hexobarbital[a] Sleeping Time (Min)	Zoxazolamine[b] Paralysis Time (Min)
None	43 ± 2[f]	102 ± 7[f]
DMSO (Vehicle)[c]	47 ± 4	88 ± 7
Malathion[d]	51 ± 6	105 ± 6
SKF 525-A[e]	167 ± 4	141 ± 2

[a] Hexobarbital dose: 320 µmoles/kg, intraperitoneally.
[b] Zoxazolamine dose: 460 µmoles/kg, intraperitoneally.
[c] DMSO dose: 1.0 ml/kg, intraperitoneally.
[d] Malathion dose: 320 µmoles/kg, intraperitoneally.
[e] SKF 525-A dose: 32 µmoles/kg, intraperitoneally.
[f] Values shown as mean ± S.E.

employed in these studies (1 ml/kg) has been reported to produce no alterations in the toxicity of several insecticides or in hexobarbital sleeping time (WEISS and ORZEL 1967). The above results differ from those of ROSENBERG and COON (1958) who reported that malathion prolonged hexobarbital sleeping time in mice; this effect has been attributed to inhibition of hexobarbital metabolism (WELCH et al. 1959). It is possible that this discrepancy is due to a species difference in the effectiveness of malathion as an inhibitor. In terms of the potential clinical significance of these observations, it should be noted that the dose of malathion employed resulted in obvious signs (salivation, fasciculations, etc.) of organophosphate intoxication. This suggests that, in the case of malathion, drug interactions attributable to the organophosphate may be expected to be of secondary importance as compared to the intoxication itself.

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