Alteration of Hepatic Microsomal Metabolism of Male Mice by certain Anticholinesterase Insecticides

by

J. T. STEVENS and F. E. GREENE

Department of Pharmacology
Milton S. Hershey Medical Center
Pennsylvania State University College of Medicine
Hershey, Pa. 17033

It is well known that many anticholinesterase insecticides serve as substrates for the hepatic cytochrome P-450 mixed function oxidase (MFO) system (O'BRIEN 1967). The components of this system include cytochrome P-450, the terminal oxidase and NADPH cytochrome P-450 reductase, more commonly referred to as NADPH cytochrome C reductase. The oxidative metabolism of drugs and other xenobiotics is thought to involve binding of the drug to the oxidized form of cytochrome P-450 (REMMER et al. 1966). After binding has occurred, the drug: P-450 complex is reduced by an electron transfer from NADPH cytochrome P-450 reductase to cytochrome P-450 (GILLETTE et al. 1972). The next step involves the reaction of the drug: reduced P-450 complex with molecular oxygen (GIGON et al. 1969). It is believed that a second electron derived from NADH or NADPH (HILDEBRANDT and ESTABROOK 1971) reduces the drug: cytochrome P-450: O_2 complex to form an active oxygen intermediate that decomposes with the formation of the product and the oxidized cytochrome P-450.

The purpose of the present paper is to provide evidence indicating that the affinity constant (Ks) for binding of parathion, disulfoton and carbaryl to mouse cytochrome P-450 is well correlated with the inhibitory constants (Ki) of these insecticides for ethylmorphine N-demethylase activity, suggesting that the inhibitory effect of these insecticides (STEVENS et al. 1972a) is associated with their ability to bind to cytochrome P-450. In addition, this study illustrates that the response seen to these insecticides after 1 hour post acute exposure, 48 hours post acute exposure, and after three and five days of daily administration (subacute) may be related to alteration in the content or functional status of cytochrome P-450.

METHODS AND MATERIALS

Adult, male Swiss-Webster mice (30 gm) were used in this study. Mice were sacrificed by cervical dislocation, their livers immediately excised, homogenized in cold 0.02 M Tris-1.15% KCl, and microsomes were prepared by differential centrifugation as previously described (GREENE et al. 1969).

The N-demethylation of ethylmorphine was determined by the method of NASH (1953). Reaction mixtures (3 ml) were incubated in a Dubnoff metabolic shaker at 37°C with air as the gaseous phase. Each mixture contained a NADPH generating

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system consisting of 3 µmoles of NADP, 40 µmoles of sodium isocitrate and 1.5 units of isocitrate dehydrogenase and 25 µmoles of magnesium chloride. Ethylmorphine (5 µmoles) and the microsomes derived from 250 mg of fresh liver were added in 0.02 M Tris-1.15% KCl at pH 7.4 to buffer the system.

Binding of the insecticides to cytochrome P-450 was determined using an Aminco-Chance dual wavelength-split beam spectrophotometer in the dual wavelength mode. Three ml of microsomes (1 mg/ml) were placed in a quartz cuvette. Microliter additions of insecticides in absolute ethanol were made to the sample cuvette. The maximum amount of ethanol added was 10 µl which did not produce significant spectral change under the conditions of these experiments. Affinity constant, (which reflects the concentration of substrate binding at one-half the ΔA_{max}) and maximal spectral change, ΔA_{max} (which indicates the relative magnitude of spectral change) were determined as the difference between the trough (420 nm) and 470 nm. These constants were determined by weighted least squares regression analysis (WILKINSON 1961). Cytochrome P-450 content was determined by measuring absorbance change between 450 and 490 nm of the CO-complex after reduction with sodium dithionite (OMURA and SATO 1964).

The rate of reduction of cytochrome C was determined by the method of WILLIAMS and KAMINS (1962) and NADPH oxidase activity by the procedure of GILLETTE et al. (1957). Both assays were conducted at 37°C in the cell block of a Gilford 2400 recording spectrophotometer. Microsomal protein content was estimated by the biuret procedure of GORNALL et al. (1949).

Highly purified insecticides were provided through the generosity of their manufacturers: disulfoton from Chemagro, Kansas City, Missouri, and carbaryl from Union Carbide, Messina, California. Parathion was purchased from Allied Sciences, State College, Pennsylvania, and Cofactors, from Sigma Biochemical Co., St. Louis, Missouri. Data were analyzed by the Student t-test.

RESULTS AND DISCUSSION

The binding constants for parathion, disulfoton, and carbaryl to cytochrome P-450 from the male mouse is given in Table 1.

It can be seen that parathion, and disulfoton which are Type I binding compounds, exhibit very similar ΔA_{max} values; however, parathion had much stronger affinity (lowest Ks) for cytochrome P-450 than the other agents. Carbaryl was found to have a larger ΔA_{max} but low affinity for cytochrome P-450. MAILMAN and HODGSON (1972) have also reported that carbaryl is a Type I binding compound in the mouse. Since there were marked differences in the affinity of these insecticides for mouse cytochrome P-450 it was of interest to see if this was reflected in their ability to inhibit the metabolism of other Type I compounds *in vitro*. The inhibitor constants (Ki) for ethylmorphine (a Type I compound) N-demethylase after the *in vitro* addition of parathion, malathion and carbaryl are given in Table 2.

TABLE 1

Binding constants for parathion, disulfoton and carbaryl to cytochrome P-450 from the male mouse

Insecticide	Binding Type	Ks (μM)	ΔA_{max} [a]
Parathion	Type I	2.77 ± 0.41	0.0066 ± 0.0005
Disulfoton	Type I	46.50 ± 9.73	0.0056 ± 0.0006
Carbaryl	Type I	259.43 ±30.08	0.0151 ± 0.0008

[a] ΔA_{max} expressed as the difference between 470 nm and the trough (420 nm) per mg of microsomal protein.

TABLE 2

Inhibitory constants (Ki) of parathion, disulfoton and carbaryl for mouse hepatic ehtylmorphine N-demethylase.

Insecticide	Ki[a] (μM)
Parathion	19.6 ± 5.2
Disulfoton	175.0 ±55.0
Carbaryl	246.7 ±27.4

[a] Ki values were determined by computer analysis using a Dixon Plot. The concentrations of insecticide examined were 0.002, 0.02, 0.2 and 1 mM. Values are means ± S.E. of 4 determinations.

These results are consistent with the previously reported inhibition of Type I compounds by anticholinesterase insecticides (STEVENS et al. 1972a, STEVENS 1973). The Ki values for ethylmorphine N-demethylase inhibition were higher than the Ks values for the binding of these insecticides to cytochrome P-450. However, the correlation coefficient between these parameters was 0.84.

Although these insecticides exhibit Type I binding spectra and inhibit the metabolism of Type I compounds, the kinetics of ethylmorphine N-demethylation by mouse microsomes

are not affected in a similar manner; ie. parathion is a competitive inhibitor and carbaryl and disulfoton are noncompetitive inhibitors (STEVENS et al. 1972a). These results suggest that the inhibition of ethylmorphine metabolism may involve more than simple competition for the terminal oxidase of the MFO system, cytochrome P-450.

It has been previously reported(STEVENS and GREENE, Submitted for publication) that the in vitro inhibition of ethylmorphine N-demethylase of rat hepatic microsomes was not well correlated with the in vitro effects of parathion, malathion, paraoxon and malaoxon on NADPH oxidation, cytochrome C reduction or the reduction of cytochrome P-450 but well correlated ($r = 0.99$) with their ability to bind to cytochrome P-450. In addition, it has been reported that mice given anticholinesterase insecticides one hour before sacrifice resulted in the inhibition of the rate of reduction of cytochrome P-450 and cytochrome C by NADPH and decreased NADPH oxidase activity and cytochrome P-450 content. However, disulfoton and carbaryl reduced the rate of reduction of cytochrome C without altering the activity of the other components of the MFO system (STEVENS et al. 1973). Inhibition of cytochrome C reduction and ethylmorphine N-demethylase activity was correlated at $r = 0.80$ (STEVENS et al. 1973).

The observation of inhibition of enzyme activity in microsomal preparations after in vivo pretreatment with an inhibitor is a function of the compound's ability to remain in the hepatic microsomes during preparative ultracentrifugation or of the ability to produce inactivation or destruction of microsomal enzymes. Such an effect is perhaps a better indicator of a compound's ability to produce significant inhibition in the intact animal, than the usual in vitro methods.

The fact that parathion and its oxygenated analog, paraoxon, reduced the level of cytochrome P-450, may be of some importance in abscribing a mode of action to parathion, since in some circumstances cytochrome P-450 content may be rate-limiting (ORRENIUS and ERNSTER 1964). Further, the strength of binding to cytochrome P-450 may be important in the inhibitory mechanism since parathion binds very strongly to cytochrome P-450 from the mouse. We have previously shown that parathion pretreatment decreased the affinity of parathion for rat cytochrome P-450 by 71% (STEVENS et al. 1973). Such an alteration suggests that cytochrome P-450 is either functional by modified or that parathion or a metabolite is still tightly bound to cytochrome P-450.

Although it has been reported that sacrificing mice, one hour after the peroral administration of parathion, resulted in inhibition of enzyme activity (STEVENS et al. 1973). A time delay of 48 hours post a single dose produced a different effect (Table 3).

Ethylmorphine N-demethylase and cytochrome P-450 content were increased to 130 and 131% of the controls, respectively. NADPH cytochrome C reductase activity was slightly elevated but NADPH oxidase activity significantly reduced. Since 1 hour after an acute dose, parathion inhibited ethylmorphine N-

TABLE 3

Effect of in vivo exposure to parathion 48 hours before sacrifice on the MFO system of the male mouse.

Group	Ethylmorphine N-demethylase[b]	NADPH Cytochrome C reductase[b]	NADPH oxidase[b]	Cytochrome P-450 content[c]
Control	7.93 ± 0.34	102.00 ± 7.02	10.42 ± 0.83	0.61 ± 0.04
Parathion[a]	10.31 ± 0.50[d]	122.67 ± 7.97	7.21 ± 0.58[d]	0.80 ± 0.09

[a] Sacrificed 48 hr post ½ LD_{50} (36.2 µmoles/kg).

[b] Activity as nmoles of substrate/mg of microsomal protein/min ± S.E. (N = 4).

[c] nmoles of cytochrome P-450/mg of microsomal protein.

[d] Different from the control at $P<0.05$.

demethylase activity, NADPH cytochrome C reductase activity and reduced cytochrome P-450 content, these results at 48 hours suggest that importance of time in the response produced. This is reminescent of biphasic response seen with hexobarbital hypnosis after SKF-525A (RUMKE and BOUT 1960).

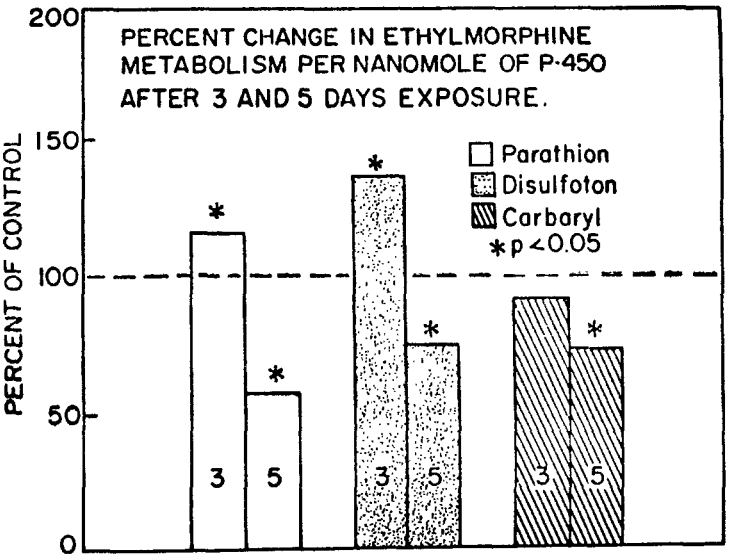
It has been reported previously that the subacute administration of many anticholinesterase insecticides resulted in the induction of hepatic drug metabolism (STEVENS et al. 1972b) and the MFO system (STEVENS et al. 1973).

Although three days of exposure to carbaryl and disulfoton was adequate to induce ethylmorphine metabolism, five days of parathion pretreatment were necessary for induction (STEVENS et al. 1973). It was noted that after the later study was published that the cytochrome P-450 induced by these insecticides after 5 days was much greater than ethylmorphine N-demethylase. Making the assumption that the ratio of ethylmorphine N-demethylase activity to cytochrome P-450 content for the controls reflects one hundred percent efficiency, then values of less than that of the controls for insecticide induced microsomes would reflect a decrease in the ability of cytochrome P-450 to metabolize ethylmorphine. This retrospective view reveals that if ethylmorphine metabolism is expressed per nanomole of cytochrome P-450, the cytochrome P-450 present in the microsomes after 5 days of exposure to these insecticides was less metabolically active than that present in the control microsomes (Figure 1).

At three days, the cytochrome P-450 induced was at least as metabolically functional as that of the controls. The reason for this transition between three and five days of subacute exposure is unclear.

It is tempting to speculate, if an insecticide is tightly bound to cytochrome P-450 and metabolized at a rate that is less than the amount administered daily, there might be a build up of the insecticide available for binding to cytochrome P-450. At the same time this bound cytochrome P-450 would not be functional in drug metabolism. This idea is not foreign to

FIGURE 1



drug metabolism since it has been shown by SCHENKMAN et al. 1972 that SKF 525-A administered in vivo, forms a stable oxygenated complex with ferrous P-450 which can survive the preparation of microsomes. In addition, certain methylenedroxyphenyl synergists have also been shown to form stable metabolite-cytochrome P-450 complexes (FRANKLIN 1971).

The synthesis of new cytochrome P-450 may then occur to compensate for the loss of P-450 due to insecticide binding. As the synthesis of P-450 exceeds the nonfunctional bound P-450, increases in ethylmorphine metabolism would be observed. The discrepancy between the ratio of ethylmorphine metabolism to cytochrome P-450 content could simply be due to the inability to distinguish between enzymatically active and enzymatically inactive (bound) cytochrome P-450 since the assay procedure usually measures total cytochrome P-450 even in the presence of substrates that bind to it.

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