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Effect of Diisopropyl Fluorophosphate on Hepatic Microsomal Systems in Two Strains of Rats

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One of the largest and most widely used classes of pesticides are the organophosphorus compounds (OP). While it is well established that the acute toxicity of OP is mediated primarily by a marked increase in acetylcholine concentration subsequent to central and peripheral cholinesterase (ChE) inhibition, there is good evidence for direct non-cholinergic effects of these compounds (Marquis 1985). It is well known that some OP containing P=S groups such as parathion, malathion or fenitrothion are transformed into active P=O ChE inhibitors by hepatic microsomal systems, i.e. they are their substrates. These compounds have been also shown to inhibit microsomal oxidation both in vitro and in vivo (Gundu Rao and Anders 1973; Uchiyama et al. 1975; Yoshida et al. 1975; Neal and Halpert 1982; Cohen 1984; Reidy et al. 1987; Bulusu and Chakravarty 1988). This may depend mainly on their role as alternative substrates or on release of reactive sulfur which binds to microsomal membranes and reduces xenobiotic metabolizing activity and cytochrome P-450 content (for references see Neal and Halpert 1982). However, it is not excluded that products of microsomal oxidation, i.e. the P=O compounds, contribute to the inhibitory effect. In fact, little is known about interactions between compounds containing P=O groups and hepatic microsomal systems. As far as the in vivo effects are concerned, Uchiyama et al. (1975) showed that dichlorvos and sumioxon (25 mg/kg ip) injected into mice did not affect N-demethylation of aminopyrine and hydroxylation of aniline 4 hr after treatment. In another study, however, repeated administration by gavage of paraoxon at 1/2 of the LD 50 (7.4 μmole/kg), corresponding to 1 mg/kg, for 5 days to mice resulted in a stimulation of hexobarbital and aniline metabolism (Stevens et al. 1972b). As regards the in vitro effects, paraoxon and malaoxon, dichlorvos and sumioxon added at 10^{-4} - 10^{-3} M concentrations did not affect microsomal metabolism of the previously mentioned substrates in liver preparations from rats and mice (Gundu Rao and Anders 1973; Uchiyama et al. 1975). Other data, however, indicate that 10^{-5} M paraoxon reduced hexobarbital and ethylmorphine metabolism and enhanced that of aniline in mouse liver microsomes (Stevens et al. 1972a). The same authors showed some species differences in the in vitro effects of 10^{-3} M paraoxon

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which inhibited hexobarbital metabolism in liver preparations from rats and mice, enhanced it in those from rabbits and dogs, and stimulated aniline hydroxylase in all these species.

There are no data in the literature on the in vivo effects of P=O compounds on xenobiotic metabolizing systems in rats. It has to be taken into account that differences in these effects may exist between strains. Such differences have been reported in the response to acute and subchronic antiChE effects of diisopropyl fluorophosphate (DFP, Russell et al. 1983; Overstreet and Russell, 1984). Strain differences in the maintenance of mixed function oxidase (MFO) activities and cytochrome P-450 content in cultured rat hepatocytes have also been described (Grant et al. 1986).

The purpose of the present experiments was to assess the effects of single and repeated treatments with DFP (a prototype of P=O irreversible antiChE agents) on hepatic microsomal aminopyrine-N-demethylase and aniline hydroxylase activities, and on the cytochrome P-450 content both in Wistar and Fischer 344 rats. In parallel experiments the in vitro effects of DFP on these microsomal activities were evaluated in liver preparations from rats of the above strains which belong to those most widely used in toxicological studies.

MATERIALS AND METHODS

DFP of analytical grade (>95%) was obtained from Fluka (Buchs, Switzerland). Young Wistar and Fischer 344 male rats weighing 295 ± 15 and 245 ± 12 g, respectively, were purchased from Charles River Italia (Calco, Como). They were allowed free access to water and standard pelleted food. In time-course experiments the animals received a single sc dose of DFP (1.6 mg/kg, in arachis oil) 24,48, 72 and 96 hr before sacrifice. Repeated treatments were performed with the first sc dose of 1.6 mg/kg and subsequent five doses of 1.1 mg/kg on alternate days, and the rats were killed 48 hr after last treatment. Control animals received the vehicle. Livers were immediately removed and 12.5% (w/v) homogenates prepared with 0.01M phosphate buffer containing 1.15% KCl, pH 7.4, using a Potter-Elvehjem homogenizer with teflon pestle. The homogenates were centrifuged at 9,000 g for 20 min at 4°C and the supernatants used for enzymatic assays. Aniline hydroxylase was measured by formation of p-aminophenol, and aminopyrine-N-demethylase by production of formaldehyde according to the methods of Mazel (1972) as previously described (Meneguz and Michalek 1987). In the experiments in which the content of cytochrome P-450 was measured 9,000 g supernatants were centrifuged at 100,000 g for 60 min to obtain the microsome fraction. Cytochrome P-450 was estimated by the carbon monoxide difference spectrum of dithionite reduced microsomes (Mazel 1972). The proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. In the experiments in vitro DFP dissolved in propylene glycol was added at 10^{-6}, 10^{-5} and 10^{-4} M concentrations to the incubation mixtures containing 9,000 g supernatants prepared from liver homogenates of untreated Wistar and Fischer rats. The vehicle was added to the controls and constant final volumes of incubation mixtures (4.0 ml for aniline

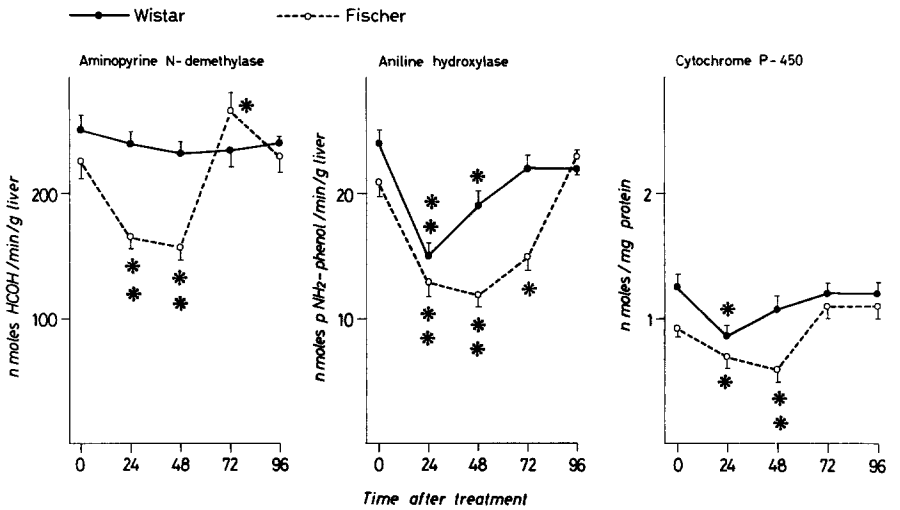

Figure 1. Time-course of the hepatic mixed function oxidase system and the cytochrome P-450 content after DFP in Wistar and Fischer rats. Animals received a single sc dose of DFP (1.6 mg/kg) and were killed after interval indicated (hr). The data on enzymatic activities were obtained on 9,000 g supernatants (microsomal proteins were 22.5±1.0 and 24.5±0.9 mg/g of liver for control Wistar and Fischer rats, respectively), those on cytochrome P-450 content on microsome preparations. Values represent mean ± SE from eight animals in each group. Significantly different from controls (**p<0.01, *p<0.05, Duncan's multiple range test)

hydroxylase and 6.0 ml for aminopyrine-N-demethylase) were always maintained; the protein content in 9,000 g supernatants was of 8.6 ± 0.2 mg, for each sample, corresponding to about 1.5 mg of microsomal protein. Statistical analyses were performed by ANOVA and Duncan's multiple range test, or Student's t-test.

RESULTS AND DISCUSSION

Body and liver weights, their ratios, as well as microsomal proteins were similar in rats of the two strains and not influenced by DFP treatment either after a single dose or after repeated doses (data not reported). The data on aminopyrine-N-demethylase, aniline hydroxylase and cytochrome P-450 content in control and DFP-treated rats are presented in Fig. 1. Although in the first experiment Wistar control rats exhibited somewhat higher levels of the two enzymatic activities with respect to Fischer rats, there were no significant strain-dependent differences between the former and the latter. With regard to DFP effects, a time-course experiment following a single dose of 1.6 mg/kg indicated that in Fischer rats at 24 and 48 hr there was a decrease (by 30 and 40% respectively, p<0.01) of aminopyrine-N-demethylase and aniline hydroxylase activities which returned to control values at 96 hr. In Wistar

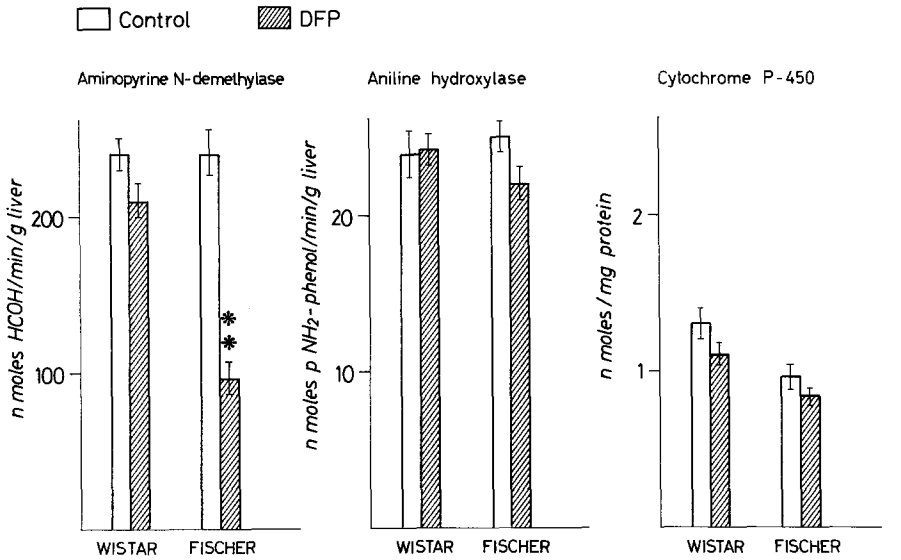

Figure 2. Effects of repeated doses of DFP on the hepatic mixed function oxidase system in Wistar and Fischer rats. Animals received sc a first dose of DFP of 1.6 mg/kg and subsequently five doses of 1.1 mg/kg on alternate days; they were killed 48 hr after last treatment. The data on enzymatic activities were obtained on 9,000 g supernatants, those on cytochrome P-450 content on microsome preparations. Values represent mean ± SE from eight animals in each group. Significantly different from control: (**p<0.01, Student's t-test)

rats the activity of aminopyrine-N-demethylase at all intervals was not modified by DFP, while aniline hydroxylase was reduced by about 30% ($p<0.01$) at 24 hr, followed by a gradual rise within 72 hr after treatment. The level of cytochrome P-450 was significantly depressed in Fischer rats at 24 and 48 hr (by 25%, $p<0.05$ and by 35%, $p<0.01$, respectively) and Wistar rats at 24 hr (by 30%, $p<0.05$). Therefore, the time-course experiment showed that after a single DFP administration the changes in enzymatic activities were most pronounced at 24 and 48 hr. The latter interval between consecutive doses was therefore used in the experiment on repeated DFP treatment.

As shown in Fig.2, repeated administration of DFP caused in Fischer rats a considerable depression of aminopyrine-N-demethylase (by about 60% $p< 0.01$), only a slight and not significant reduction of aniline hydroxylase (by about 15%), and no changes in the cytochrome P-450 content. Neither of these microsomal enzymatic activities nor the cytochrome P-450 content were affected by the same treatment in Wistar rats.
As shown in Table 1, in vitro addition of DFP (up to 10^{-4} M concentration) to liver preparations did not significantly influence

Table 1. In vitro effects of DFP on the hepatic mixed function oxidase system in Wistar and Fischer rats.

Strain	DFP M	Aminopyrine-N-demethylase	Aniline hydroxylase
Wistar	0	220 ± 8.6	23 ± 1.0
	10^{-6}	230 ± 6.6	24 ± 2.5
	10^{-5}	217 ± 9.7	24 ± 2.0
	10^{-4}	220 ± 5.7	23 ± 1.2
Fischer	0	234 ± 18.3	22 ± 0.6
	10^{-6}	211 ± 10.0	23 ± 1.6
	10^{-5}	204 ± 13.5	20 ± 1.8
	10^{-4}	196 ± 14.5	19 ± 1.5

The data were obtained on 9,000 g supernatants. Aminopyrine-N-demethylase activity is expressed as nmol of HCOH formed min/g of liver, that of aniline hydroxylase as nmol of p-aminophenol formed/min/g of liver. Values represent mean ± SE from five animals for each concentration

aminopyrine-N-demethylase and aniline hydroxylase from either Wistar or Fischer rats. As assessed by variance analysis, however, in the latter strain there was a trend toward the depression of the two enzymes which did not reach the level of statistical significance. These data are in good agreement with those previously reported on dichlorvos and sumioxon in the mouse (Uchiyama et al. 1975), and with those on paraoxon and malaoxon in the rat (Gundu Rao and Anders 1973). They differ, however, from the findings of Stevens et al. (1972a) indicating a stimulatory effect of paraoxon at 10^{-5} M concentration on aniline hydroxylase and an inhibitory effect on ethylmorphine N-demethylase in mouse microsomes.

The overall in vivo data show that the injection of DFP into rats exerts toxic effects on the hepatic xenobiotic metabolizing systems These effects consist substantially in a transitory reduction of MFO and appear more pronounced after a single dose than after a repeated treatment. Following a single DFP administration, in fact, the aminopyrine-N-demethylase, aniline hydroxylase, and cytochrome P-450 content are significantly depressed (except the first activity, in Wistar rats only). The maximum reduction occurs at 24-48 hr and is followed by a normalization of the pattern within 72-96 hr. Consecutive treatments, performed at the maximum inhibitory effects of previous ones, enhance only the depression of aminopyrine-N-demethylase in Fischer rats. On the other hand, the inhibitory effects of DFP on aniline hydroxylase and the cytochrome P-450 content disappear in all rats after repeated treatment. The differences in the response of aminopyrine-N-demethylase and aniline hydroxylase may depend on the interaction of the enzyme with the two substrates. It is well known that aminopyrine is a type I substrate and aniline a type II substrate, and that they therefore

differ in their binding to heme iron of cytochrome P-450, indirect and direct, respectively (Weiner 1985; Krainev et al. 1988).

The observed effects may be produced by the P=O anticholinesterase moiety or by the fluoride moiety of DFP. It is well known from many years that in the rat liver DFPase is present (Mounter 1963; Ramachandran and Agren 1964). This enzymatic activity hydrolyzes DFP into diisopropylphosphate and fluorides. However, it appears unlikely that the reduction of hepatic mixed function oxidase system and cytochrome P-450 content due to DFP treatment depend on fluorides release. In fact, no effects of prolonged fluoride exposure on liver monooxygenases and cytochrome P-450 have been observed in rats (Hongslo et al. 1983). Similarly, mixed function oxidase activities such as ethylmorphine demethylation and benzopyrene hydroxylation in rat liver microsomes were not affected by fluorides (Post and Snyder 1983). Therefore the observed effects appear to be produced by the P=O anticholinesterase moiety rather than by fluorides.

As already mentioned, the data show some strain-differences in the response to DFP. Generally speaking, the effects observed in Fischer rats are more pronounced and longer lasting than those in Wistar rats. The difference is particularly evident in the case of aminopyrine-N-demethylase, markedly depressed in the former strain after a single DFP administration (and even more after repeated treatment) but not affected in the latter. This implies that Fischer rats exhibit more vulnerability to DFP effects on xenobiotic metabolizing systems than Wistar rats. It is of interest that the former strain is also more vulnerable to aging. Other experiments in this laboratory showed considerable age-related reduction of aminopyrine-N-demethylase, aniline hydroxylase and cytochrome P-450 in 24-month Fischer but not Wistar rats (unpublished data). Similarly, recent investigation on brain cholinergic systems in aging indicated strain-related reduction of choline acetyltransferase and muscarinic receptors in the cerebral cortex of Fischer but not age-matched Wistar senescent rats (Michalek et al. 1989).

As mentioned in the introduction, the in vivo effects of P=O compounds on MFO were studied only in mice. Both the findings of Stevens et al (1972b) on paraoxon in the mouse and the present data on DFP in the rat were obtained in animals severely intoxicated in terms of cholinergic overstimulation (1/2 of LD50 in the former, 1/3 of LD50 in the latter, resulting in about 80% of brain ChE inhibition). A stimulatory effect of paraoxon on hexobarbital and aniline metabolism was demonstrated in mice while inhibitory effects (or no changes) due to DFP on aniline and aminopyrine metabolism were detected in rats. This suggests that there exist some species differences in the in vivo response of MFO to P=O compounds. It is of interest that similar species differences have been demonstrated recently in this laboratory in the response to ethylenethiourea, a metabolite of dithiocarbamate. This compound stimulated microsomal systems in mice but not in rats (Meneguz and Michalek 1986, 1987).
Though the mechanism of MFO inhibition caused by a single in vivo treatment with DFP is obscure, it cannot be attributed to substrate

competition since a P=O compound is not an alternative substrate, unlike P=S compounds. DFP is a lipophilic compound and its incorporation into microsomal membranes may induce chemical and physical changes which alter their native properties. It has been reported that variations in microsomal lipid composition, especially an increase in the cholesterol/phospholipid ratio, may result in changes in membrane fluidity. This is presumed to alter the activity of microsomal enzymes. It is of interest that DFP has been shown to cause some alterations in the lipid composition of electroplax (from the electric eel) by raising the above mentioned ratio (Hubbard and Rosenberg 1985). Most data obtained on repeated treatment indicate that the hepatic endoplasmic reticulum compensate the damage induced by DFP. The mechanisms responsible for this compensation are again unknown but seem to be more efficient in Wistar rats.

The present study indicates that a P=O compound injected into rats induces a transitory reduction of the xenobiotic metabolizing systems. Therefore possible interactions between exposure to P=O pesticides and intake of drugs have to be taken into account.

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