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EFFECT OF PRETREATMENT WITH SODIUM PHENOBARBITAL ON THE TOXICITY OF SOMAN IN MICE*

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Abstract—Pretreatment with sodium phenobarbital induces hepatic microsomal enzymes which are responsible for the metabolic breakdown of a large number of endogenous and exogenous chemical compounds. A previous study [K. P. DuBois and F. K. Kinoshita, *Proc. Soc. exp. Biol. Med.* **129**, 699 (1968)] reported that phenobarbital pretreatment reduced the toxicity of various organophosphorus anticholinesterases; however, the exact mechanism for the increased detoxification was not investigated. In this study, the effect of phenobarbital pretreatment on the toxicity of soman was investigated. Male mice were injected daily for 4 days with sodium phenobarbital (100 mg/kg, i.p.) and used in the various experiments 24 hr after the last injection. Phenobarbital pretreatment produced a significant increase in liver weight and decreased the sodium pentobarbital (75 mg/kg, i.p.) induced sleep-time to 41 min compared to 141 min in controls. The lethality of soman was reduced following phenobarbital pretreatment. In control mice, the soman 24 hr LD_{50} values (μg/kg) were 130, 393 and 42 following s.c., i.p. and i.v. administration, respectively, whereas in phenobarbital-pretreated mice the soman 24 hr LD_{50} values (μg/kg) were 261, 746 and 63 following s.c., i.p. and i.v. administration respectively. Acetylcholinesterase activity was increased in the plasma (90%) but not in brain or diaphragm following phenobarbital pretreatment. Liver somanase activity was not affected. Liver aliesterase and serum aliesterase were both increased significantly following phenobarbital pretreatment. An increase in the amount of nonspecific binding sites for soman (esterases in liver and plasma) and not an increase in the metabolism of soman *in vivo* probably accounts for the protection afforded by phenobarbital pretreatment in mice.

A large number of drugs and xenobiotics are metabolized by enzymes present in liver microsomes. Many studies over the past two decades have demonstrated that hepatic microsomal enzymes are increased when animals are pretreated for periods of time with various drugs, insecticides, hormones and carcinogens. "This increase in activity appears to represent an increased concentration of enzyme protein and is referred to as 'enzyme induction'" [1]. Induction of hepatic microsomal enzyme activity is characterized by increases in liver weight, smooth endoplasmic reticulum, microsomal protein concentration, and metabolic activities of a number of model enzyme systems (aniline hydrolase, hexobarbital oxidase, ethylmorphine *N*-demethylase, etc.) and decreases in the sleep-times of zoxazolamine and other short-acting barbiturates such as hexobarbital and sodium pentobarbital.

Generally, if drugs are metabolized by enzymes present in the liver, the duration of action and the acute toxicity of a drug are reduced following induction of hepatic microsomal enzymes. Previous investigators have shown that induction of hepatic microsomal enzymes by pretreatment of mice with sodium phenobarbital, a potent inducer of hepatic microsomal enzymes, reduces the toxicity of a variety of insecticides [2], suggesting an increased detoxification *in vivo*. In addition, pretreatment of mice with various chlorinated hydrocarbons attenuates the acute toxicity of several organophosphorus pesticides [3]. Similarly, certain hormones are known to affect the toxicity of pesticides. DuBois *et al.* [4] noted that the sex difference in susceptibility to parathion poisoning in rats tends to disappear when male and female rats are injected with diethylstilbestrol or testosterone respectively. Various catatoxic steroids (ethylestrenol, spironolactone and norbolethone) reduce the toxicity of organophosphorus pesticides [5, 6]. This protective effect is due to induction of hepatic microsomal enzymes [7].

The purpose of this study was to investigate the mechanism involved in the reduction of soman toxicity provided by phenobarbital pretreatment of mice.

METHODS

Materials

Soman was synthesized by the Organic Chemistry Group, Defence Research Establishment Suffield. The soman was 97–99% pure as determined by the Schoeneman reaction [8]. Fresh soman solutions were prepared immediately before use by diluting the neat material in 0.9% saline to the required concentration. Sources of other drugs used were as follows: sodium phenobarbital (Fisher), sodium pentobarbital (Nembutal; Abbott); tributyrin (Fisher); and [^{14}C]acetylcholine iodide (4.0 mCi/mmole) (New England Nuclear).

Toxicology

Male CD-1 mice (25–30 g) were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec, Canada. The animals were

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acclimatized for at least 1 week following their arrival at Defence Research Establishment Suffield prior to use.

Mice were injected daily for 4 days with sodium phenobarbital (100 mg/kg, i.p.) dissolved in 0.9% saline and were used in experiments 24 hr after the last injection. In the toxicological studies, soman was diluted with 0.9% saline and injected either subcutaneously (s.c.), intraperitoneally (i.p.) or intravenously (i.v.). The injection volume of soman was 1% of body weight. Ten animals per dose and at least five different doses were used in constructing the LD_{50} curves. Twenty-four hour LD_{50} determinations and 95% confidence limits were calculated by the method of Miller and Tainter [9] using a computer program. All animals had access to food and water *ad lib.* before and after injection with soman.

Induction of hepatic microsomal enzymes was assessed indirectly by an increase in the liver to body weight ratio and a decrease in the sodium pentobarbital sleep-time. Sodium pentobarbital (75 mg/kg) was injected i.p., and the time between losing and regaining the righting reflex was noted as the sleep-time.

Acetylcholinesterase

Acetylcholinesterase activity was determined using the radiometric procedure of Siakotos *et al.* [10] using [^{14}C]acetylcholine (ACh) iodide as the substrate. The reaction was carried out at 37° in all cases.

Brain. Mice were decapitated and exsanguinated. The brain minus the cerebellum was rinsed in ice-cold saline, blotted dry on filter paper and weighed, and a 1% (w/v) homogenate was prepared in a 0.1 M phosphate buffer (pH 7.4) containing 0.4 M sucrose. The time of incubation with [^{14}C]ACh was 10 min. Acetylcholinesterase activity was expressed as nmoles ACh hydrolysed per mg wet weight of tissue per min. There was no difference in protein concentrations; therefore, results were expressed as per mg wet weight of tissue.

Diaphragm. Mice were decapitated and exsanguinated. The entire diaphragm was removed and placed in ice-cold saline. The diaphragms were blotted dry, weighed, and frozen in liquid nitrogen. They were then pulverized, and the powder was transferred to a test tube. Enough 0.1 M phosphate buffer (pH 7.4) containing 0.4 M sucrose was added to make a 10% (w/v) solution that was homogenized for 1 min in the cold using an Ultra-Turrax homogenizer (Tekmar Inc.). Each homogenate was prepared by pooling the entire diaphragms from five mice. The time of incubation with [^{14}C]ACh was 5 min. Acetylcholinesterase activity was expressed as nmoles ACh hydrolysed per mg protein per min.

Plasma. Mice were decapitated. Whole blood was collected in a plastic beaker containing a drop of heparin (1000 units/ml) and centrifuged, and the plasma was drawn off. Plasma (10 μl) was incubated with [^{14}C]ACh for 5 min. Acetylcholinesterase activity in plasma was expressed as nmoles ACh hydrolysed per ml plasma per min.

Aliesterase

Aliesterase activity was assessed by the pH-stat technique using tributyrin as the substrate. To a reaction vessel (23°) tributyrin (10 ml of 0.2% solution) was added, and the solution was titrated to pH 7.9 using a Radiometer titragraph. A 10% (w/v) liver homogenate was prepared using 0.9% saline in a Teflon glass homogenizer. To start the reaction either 50 μl of serum or 25μl of a 10% (w/v) liver homogenate was added. Rate of addition of 0.01 N NaOH from the first to fifth minute was used in determining enzyme activity. The reaction was linear over this time period. All solutions were degassed by bubbling pure nitrogen through them for at least 5 min, and the reaction vessel was purged with nitrogen while the reaction was in progress.

Somanase

Somanase activity was determined by the pH-stat method. A 10% (w/v) liver homogenate was prepared using 0.9% saline in a Teflon glass homogenizer. A 10 ml sample of soman (1.1 mM in 0.9% saline) was placed in a vessel, and the pH was titrated to 7.2 at 23° with NaOH (0.01 N). Then a 400 μl sample of a 10% (w/v) liver homogenate was added to start the reaction. The consumption of NaOH over a 5-min period was used in calculating somanase activity. The reaction was linear over this time period. All solutions were degassed by bubbling pure nitrogen through them for at least 5 min, and the reaction vessel was purged with nitrogen while the reaction was in progress.

RESULTS

Pretreatment of mice with sodium phenobarbital (100 mg/kg) for 4 days decreased significantly ($P < 0.001$) the sodium pentobarbital-induced sleep-time (Table 1) and increased significantly ($P < 0.001$) the liver/body weight ratio. These two variables indicate, indirectly, induction of hepatic microsomal enzymes.

The effect of phenobarbital pretreatment on the toxicity of soman was assessed (Table 2). Phenobarbital pretreatment decreased significantly the toxicity of soman by the s.c., i.p. and i.v. routes of administration. The LD_{50} value of soman following phenobarbital pretreatment was increased 100, 90 and 50% following s.c., i.p. and i.v. administration respectively.

The activities of acetylcholinesterase in plasma, brain and diaphragm were assessed using the procedure of Siakotos *et al.* [10]. The results in Table 3 indicate that phenobarbital pretreatment had no significant effect on the acetylcholinesterase activities in brain and diaphragm. In contrast, phenobar-

Table 1. Effect of phenobartital pretreatment on sodium pentobarbital sleep-time and liver weight*

Treatment	Sleep-time (min)	Liver weight (% of body weight)
Control	141 ± 37 (50)	6.14 ± 0.34 (10)
Phenobarbital	41 ± 16† (50)	7.9 ± 0.54† (30)

* Values are expressed as means ± S.D. (N). Sodium pentobarbital (75 mg/kg) was injected i.p.

† Significantly different from control group ($P < 0.001$).

Table 2. Effect of phenobarbital pretreatment on the toxicity of soman in mice

	Soman LD_{50} (μg/kg)					
	Route					
Treatment	s.c.	% increase	i.p.	% increase	i.v.	% increase
Control	130* (116–146)		393 (366–417)		42 (39–46)	
Phenobarbital	261 (245–289)	100	746 (682–828)	90	63 (59–67)	50

* LD_{50} (95% confidence limits).

Table 3. Effect of phenobarbital pretreatment on acetylcholinesterase activity in mice*

	Acetylcholinesterase activity		
Treatment	Plasma (nmoles ACh·ml^{-1}·min^{-1})	Brain [nmoles ACh·(mg brain tissue)$^{-1}$·min^{-1}]	Diaphragm [nmoles ACh·(mg protein)$^{-1}$·min^{-1}]
Control	1131 ± 61 (10)	11.6 ± 0.25 (10)	7.18 ± 0.74 (5)
Phenobarbital	2149 ± 95† (8)	11.1 ± 0.30 (7)	7.38 ± 0.67 (6)

* Values are expressed as means ± S.E.M. (N).
† Significantly different from control value ($P < 0.001$) as determined by Student's t-test.

Table 4. Effect of phenobarbital pretreatment on liver somanase activity*

Treatment group	Somanase activity (nmoles soman hydrolysed·g^{-1}·min^{-1})
Control	616 ± 48 (4)
Phenobarbital	556 ± 81 (7)

* Values are expressed as means ± S.D. (N).

bital pretreatment produced a 90% increase in plasma acetylcholinesterase activity.

The activity of an A-esterase somanase (phosphorylphosphatase), an enzyme which hydrolyses soman but is not inhibited by it, was examined in liver of phenobarbital-pretreated mice. The results in Table 4 indicate that liver somanase activity in phenobarbital-pretreated mice was not significantly different from that in control mice.

Aliesterase, a B-esterase which is inhibited by organophosphates, is important in the detoxification of soman. Aliesterase activity was examined in control and phenobarbital-pretreated mice using tributyrin as the substrate. The results in Table 5 show that phenobarbital pretreatment increased significantly ($P < 0.001$) aliesterase activity in both serum and liver.

DISCUSSION

Phenobarbital is a model inducer of hepatic microsomal enzymes in mice. Induction of hepatic microsomal enzymes increases the metabolism of drugs and various xenobiotics and thus decreases their half-lives in the body and their acute toxicities. There are, however, special instances where xenobiotics are activated, i.e. are metabolized to more potent compounds, by the hepatic microsomal enzyme system (e.g. phosphorothionate insecticides).

Pretreatment of animals with phenobarbital [2, 11, 12], chlorinated hydrocarbons [3, 13–15], or hormones [5] reduces the toxicity of a number of organophosphorus anticholinesterase compounds. In general, the reduction in the toxicities of the organophosphates has been thought to result from increases in the oxidation of the organophosphorus

Table 5. Effect of phenobarbital pretreatment on aliesterase activity in liver and serum*

	Aliesterase activity			
Treatment	Serum (nmoles TBT hydrolysed·ml^{-1}·min^{-1})	% increase	Liver [nmoles TBT hydrolysed·(mg protein)$^{-1}$·min^{-1}]	% increase
Control	2496 ± 167 (8)		607 ± 100 (10)	
Phenobarbital	3850 ± 450† (9)	54	900 ± 139† (9)	48

* Values are expressed as means ± S.D. (N). TBT = tributyrin.
† Significantly different from the control group ($P < 0.001$).

compounds by the hepatic microsomal enzymes [3, 7]. In the present study, phenobarbital pretreatment decreased the toxicity of soman following s.c., i.p. or i.v. administration. However, an increase in the oxidative metabolism of soman in the liver is doubtful, since no oxidative enzymes seem to metabolize soman. The metabolism of soman occurs via hydrolysis, forming hydrofluoric acid and pinacolyl methylphosphonic acid, and soman does not appear to be further oxidatively dealkylated to methylphosphonic acid [16]. Liver somanase (phosphorylphosphatase) is an A-esterase [17] which hydrolyses soman. However, soman comprises four stereoisomers, and somanase hydrolyses the two least toxic isomers [18]. Similarly, rat plasma phosphorylphosphatase hydrolyses the least toxic isomer of sarin [19]. Induction of hepatic microsomal enzymes by phenobarbital pretreatment was indicated indirectly by a decrease in sodium pentobarbital-induced sleep-time (Table 1) and a decrease in soman toxicity (Table 2). However, the hydrolysis of soman by liver somanase was not increased following phenobarbital pretreatment (Table 4). In addition, CBDP (2-/*o*-cresyl/-4*H*-1:3:2-benzodioxa-phosphorin-2-oxide) pretreatment, which inhibits plasma aliesterase and cholinesterase, increases the toxicity of soman 16–19 times (unpublished observations) [20], but has no effect on liver somanase activity (unpublished observations*). Fasting decreases the activity of hepatic microsomal enzymes [21]; however, in CD-1 mice the soman LD_{50} is not affected by an 18-hr fast period [22], suggesting that detoxification of soman by the liver following s.c. administration is not a major route of metabolism. In the present study, following phenobarbital pretreatment the toxicity of soman was reduced irrespective of the route of administration, suggesting that the first-pass through the liver was not the primary reason for the reduced toxicity of soman. Based on the above observations, it is highly unlikely that the effect of phenobarbital to protect against soman toxicity was due to an increase in the hydrolysis of soman by liver somanase.

An increased metabolism of soman by microsomal enzymes in extrahepatic tissues is doubtful since phenobarbital is a poor inducer of these extrahepatic enzymes [23]. The present study, however, shows that in phenobarbital-pretreated mice, increases occur in other extrahepatic enzymes, e.g. plasma aliesterase and plasma acetylcholinesterase (which is primarily due to pseudocholinesterase activity [24]). This apparent anomaly is probably due to the fact that pseudocholinesterase [25] and perhaps aliesterase [26] are synthesized in the liver and are secreted into the blood. Phenobarbital pretreatment also produced an increase in liver aliesterase (Table 5).

Detoxification of a drug is not synonymous with metabolism to a less toxic compound. Detoxification could result from binding to proteins or by storage in a lipid compartment, thus leaving little of the free drug to combine with specific sites responsible for the toxic and lethal effects of a drug. Sarin [27, 28] and diisopropyl fluorophosphate (DFP) [29] bind to plasma and red blood cell aliesterase to a large extent, and only a very small portion of the administered dose is actually used to inhibit acetylcholinesterase [29, 30]. Myers [31] concluded that most of the sarin administered to kill a rat is actually used up by the inhibition of aliesterase. Aliesterase is a B-esterase [17] which hydrolyses both aromatic and aliphatic *uncharged* carboxylic esters and does not hydrolyse acetylcholine and similar substrates [32]. The importance of aliesterase in organophosphate detoxification *in vivo* is demonstrated by the fact that CBDP, which is a potent irreversible inhibitor of aliesterase when combined with atropine, increased dramatically the toxicity of soman (unpublished observations) [20], whereas, carbamates, which do not have a great affinity for aliesterase [33], when combined with atropine decrease significantly the toxicity of soman [34]. The lethality of *O*-ethyl-*S*-(2-diisopropylaminoethyl)-methylphosphorofluoridate (VX), which is not supposed to bind to nonspecific sites to any great degree, was not affected significantly by CBDP pretreatment [20]. Near complete inhibition of only plasma cholinesterase by tetraisopropyl phosphoramide (iso-OMPA) had no effect on soman toxicity in mice (unpublished observations).

Therefore, like sarin [31], most of the administered soman may be detoxified by aliesterase in the plasma and liver. This hypothesis is supported by the experimental findings in phenobarbital-pretreated mice which demonstrated that plasma and liver esterases were increased significantly and that the toxicity of soman was reduced irrespective of the route of administration. The differences in the soman LD_{50} values following the different routes of administration were perhaps due to different detoxifying effects of the various esterases present in the circulatory system and the liver. These plasma and liver esterases could form the nonspecific sites [20] or they could be the soman depot(s) alluded to by other investigators [18, 35–38].

It appears that the decrease in the toxicity of soman following phenobarbital-pretreatment was due to an increased binding of soman to aliesterase in plasma and liver. Aliesterase is very important in detoxifying the majority of soman which is administered to the mice. This conclusion is similar to that of Fonnum and Sterri [39].

* Bošković [20], in his study on CBDP, measured somanase activity and not aliesterase activity as he stated, since he used soman as a substrate in his enzyme system. He also found that CBDP did not inhibit somanase activity.

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