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INDUCTIVE EFFECTS OF 1,1,1-TRICHLORO-2,2-BIS(*p*-CHLOROPHENYL)ETHANE(DDT), PHENOBARBITAL, AND BENZPYRENE ON MICROSOMAL CYTOCHROME P_{450}, ETHYL ISOCYANIDE SPECTRA, AND METABOLISM *IN VIVO* OF ZOXAZOLAMINE AND HEXOBARBITAL IN THE MOUSE*

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Abstract—Significant decreases in zoxazolamine paralysis and hexobarbital sleeping times were observed in mice following pretreatment with DDT, phenobarbital or benzpyrene. In parallel experiments, highly significant elevations were noted in the cytochrome P_{450} level and the 430 and 455 nm peaks of the ethyl isocyanide spectra of cytochrome P_{450}. These increases greatly exceeded those increases observed for liver wet weight and microsomal protein. No shifts in the ethyl isocyanide pH equilibrium point were noted when mice were pretreated with DDT, phenobarbital or benzpyrene.

In contrast to experiments reported on other mice, DDT appears to be a non-selective inducer in the strain examined.

THE HEPATIC endoplasmic reticulum of mammals is responsible for the metabolism of various xenobiotics. Many of these compounds, such as drugs, insecticides and polycyclic hydrocarbons, modify the activity of the microsomal enzyme system, and pharmacologically significant effects have been noted.[1] These compounds mediate their effects by increasing the level of the components of the microsomal oxidase system; for example, it has been reported that NADPH cytochrome c reductase activity, the level of cytochrome P_{450}, and oxidative demethylation all increase after pretreatment with phenobarbital.[2]

One component of this oxidative system, cytochrome P_{450}, was first described in 1958.[3,4] Subsequently, it was demonstrated that cytochrome P_{450} was the terminal oxidase in the microsomal drug metabolizing system.[5] Omura and Sato[6] reported that this cytochrome is a hemoprotein and when reduced binds with CO to give an absorption peak at 450 nm. The reduced cytochrome also combines with ethyl isocyanide, but the resulting spectrum contains two Soret peaks rather than the usual one. This difference was examined further by Imai and Sato[7] who reported that the relative heights of the two peaks, occurring at 430 and 455 nm, were greatly affected by pH changes. Using rabbit liver microsomes, they showed that only the 430 nm peak was present at pH 6·0, while at pH 8·0, the 455 nm peak was the predominate one. At pH 7·4, the two peaks were of equal magnitude, and this pH was described as the ethyl isocyanide equilibrium point.

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Sladek and Mannering[8–10] showed that pretreatment with 3-methylcholanthrene shifted the ethyl isocyanide pH equilibrium point from 7·4 to 6·9 while phenobarbital although increasing the size of both peaks did not produce a pH shift. The effect of 3-methylcholanthrene could be attributed to a rise in the level of the 455 nm peak while the 430 nm peak remained at the control level. These differences between phenobarbital and 3-methylcholanthrene were reflected in their effect on the metabolism of ethylmorphine (EM) and 3-methyl- 4-monomethylaminoazobenzene (3-MMAB). Pretreatment with phenobarbital resulted in an increase in the metabolism of both substrate types which was proportional to the increase in the 430 and 455 nm peaks. However, 3-methylcholanthrene caused an increase only in the metabolism of 3-MMAB while the rate for EM metabolism remained at the control level as did the 430 nm peak. One possible explanation given by Sladek and Mannering[8–10] was that the 455 nm peak was associated with the metabolism of 3-MMAB while the 430 nm peak paralleled the metabolism of EM. They also suggested that 3-methylcholanthrene may induce the formation of a new cytochrome, P_{1-450}. These postulations could account for the fact that 3-methylcholanthrene, which stimulates the metabolism of type II substrates, is a more selective inducer than phenobarbital. This effect of 3-methylcholanthrene was confirmed by Alvares *et al.*[11]

The present study was undertaken to determine the effects of pretreatment with DDT, phenobarbital and benzpyrene, a compound similar in action to 3-methylcholanthrene,[1] on metabolism *in vivo* of hexobarbital and zoxazolamine. Parallel experiments were made to determine the effects of these compounds on cytochrome P_{450} levels, ethyl isocyanide spectra, and equilibrium points.

METHODS

Five-week-old male mice obtained from the North Carolina Department of Health, an inbred colony maintained since 1910, were used for these experiments. The mice were fed water and Purina Lab Chow *ad lib.* and were housed in plastic cages using San-I-Cel bedding for litter.

All experimental compounds were given by interperitoneal injection (i.p.) using methoxytriglycol as the carrier for benzpyrene (15 mg/kg) and DDT (100 mg/kg) and distilled water for phenobarbital (100 mg/kg). The dose was delivered in 0·0025 ml solution per g of animal weight. Each experimental group received daily injections of the test chemical for 3 days, and control mice received the solvent only.

Hexobarbital sleeping times (HST) of pretreated and control mice were determined after the administration of 125 mg/kg (i.p.) hexobarbital sodium in distilled water. The duration of the zoxazolamine paralysis time (ZPT) was determined after the injection of 100 mg/kg (i.p.) zoxazolamine in methoxytriglycol. HST and ZPT were measured from the onset of sleep or paralysis, respectively, until the time the mice were able to "right" themselves twice within 15 sec. Eight mice were used for each treatment and control group.

For cytochrome P_{450} and ethyl isocyanide spectral analyses, the mice were sacrificed, livers removed and rinsed in buffer. They were then placed in ice-cold 0·15 M KCl-50 mM tris-HCl buffer (pH 7·4). Subsequently, the livers were blotted, weighed and homogenized in 4 vol. of the KCl–tris-HCl buffer (4 ml:g wet wt.). Two livers were pooled in each test. The homogenate was centrifuged at 10,000 *g* for 15 min

and the microsomal fraction sedimented by spinning the post-mitochondrial supernatant at 100,000 *g* for 1 hr. The resulting supernatant was discarded and the pellet suspended in 50 mM tris-HCl buffer (pH 7·5) (2 ml:g wet wt. liver). Spectral analyses of this suspension showed no evidence of hemoglobin and insignificant amounts of cytochrome P_{420}.

Ethyl isocyanide was synthesized using the method of Jackson and McKusick.[12] For the determination of the ethyl isocyanide spectra, 0·5-ml samples of the microsomal suspension were diluted wth 5 ml of 0·5 M phosphate buffer at the desired pH. The suspensions were reduced with dithionite and base lines established. After addition of ethyl isocyanide to 15 mM in the sample cuvette, the spectra were recorded and the pH of the samples rechecked. Initial ethyl isocyanide curves were determined from pH 6·5 to 8·0. Subsequent experiments were limited to those pH values closest to the equilibrium points. Cytochrome P_{450} levels were determined using 0·5-ml samples of the microsomal suspension diluted with 5 ml of the 50 mM tris-HCl buffer (pH 7·5) according to the method of Omura and Sato.[6] All spectra were obtained using a Beckman DK-2 ratio recording spectrophotometer. Each experiment was replicated twice, and the averages of the two tests are reported.

Protein was determined by the method of Lowry *et al.*[13] using bovine serum albumin as a standard.

Student's *t*-test was utilized to determine differences in the sleeping and paralysis time experiments. The 0·05 level was selected to establish significance.[14]

RESULTS

Pretreatment of mice with DDT, phenobarbital or benzpyrene significantly decreased both zoxazolamine paralysis and hexobarbital sleeping times (Table 1).

These inductive effects on metabolism *in vivo* were reflected in parallel increases in the cytochrome P_{450} levels and both the 430 and 455 nm peaks of the ethyl isocyanide spectra (Table 2). Accordingly, no pH shift of the ethyl isocyanide equilibrium point was produced by any of these compounds (Table 2).

TABLE 1. EFFECTS OF DDT, PHENOBARBITAL OR BENZPYRENE PRETREATMENT ON ZOXAZOLAMINE PARALYSIS TIME AND HEXOBARBITAL SLEEPING TIME IN MICE

Pretreatment*	Zoxazolamine paralysis time	Hexobarbital sleeping time†‡
DDT	12 ± 4	29 ± 6
Control	52 ± 11	53 ± 13
Phenobarbital	4 ± 3	12 ± 2
Control	56 ± 8	41 ± 12
Benzpyrene	8 ± 2	20 ± 4
Control	56 ± 8	41 ± 12

* Treated mice received DDT (100 mg/kg), phenobarbital (100 mg/kg) or benzpyrene (15 mg/kg) daily for 3 days prior to final treatment of zoxazolamine (100 mg/kg) or hexobarbital (125 mg/kg) on day four.

† Time reported in minutes ± standard deviation.

‡ Differences significant at the 0·05 level.

TABLE 2. EFFECT OF PRETREATMENT WITH DDT, PHENOBARBITAL OR BENZPYRENE ON LIVER WET WEIGHT, MICROSOMAL PROTEIN, CYTOCHROME P_{450} AND ETHYL ISOCYANIDE P_{450} SPECTRA

Treatment*	Liver wet weight	Microsomal protein (mg)	Mg microsomal protein/mg wet weight	Cytochrome P_{450}	Per cent of control (O.D./1 mg protein per ml)† Ethyl isocyanide spectra pH 7·5 430 peak	pH 7·5 455 peak	pH 7·75 430 peak	pH 7·75 455 peak	Ethyl isocyanide equilibrium point (pH)‡
DDT	1·303	27·0	0·021	210	198	225	212	210	7·66 ± 0·01
Phenobarbital	1·289	25·2	0·020	244	232	262	285	271	7·67 ± 0·03
Benzpyrene	1·202	24·5	0·021	158	153	180	175	170	7·64 ± 0·01
Control	1·177	23·5	0·020	100	100	100	100	100	7·68 ± 0·03

* Treated mice received DDT (100 mg/kg), phenobarbital (100 mg/kg) and benzpyrene (15 mg/kg) daily for 3 days and were sacrificed on the fourth day.

† Control levels (O.D./mg/cc): 450 nm—0·069; pH 7·5, 430 nm—0·057, 455 nm—0·040.
pH 7·75, 430 nm—0·041, 455 nm—0·048.

‡ Standard error of the mean of two determinations.

In addition, pretreatment led to an increase in microsomal protein which in all cases was directly proportional to an increase in liver wet weight (Table 2). However, the inductive effect of these compounds on the level of cytochrome P_{450}, the ethylisocyanide Soret peaks, and metabolism *in vivo* was much greater than the observed increase in microsomal protein (Table 2). These results preclude general liver proliferation as a primary cause of the increases noted.

DISCUSSION

Stimulation of the metabolism of both hexobarbital and zoxazolamine by DDT and benzpyrene was unexpected. Cram and Fouts,[15] using the Swiss–Webster strain of mice, had previously reported that DDT was a more selective inducer of microsomal mixed function oxidases than chlordane or phenobarbital as DDT lowered zoxazolamine paralysis time but not hexobarbital sleeping time. Similarly, benzpyrene lowered the zoxazolamine paralysis time but not the hexobarbital sleeping time in mice.[16]

With the strain of mice used in these experiments, all three compounds raised the levels of both the 430 and 455 nm Soret peaks of the ethylisocyanide spectra, a result expected of compounds that stimulate the metabolism of both type I (hexobarbital) and type II (zoxazolamine) substrates. In rats, Sladek and Mannering[8–10] reported that phenobarbital raised both ethyl isocyanide Soret peaks and metabolism of type I and II substrates, whereas 3-methylcholanthrene raised only the 455 peak and concomitantly only the metabolism of 3-MMAB, a type II substrate.

Two possible explanations could account for the results presented in this study: first, the strain of mice used for these experiments has only one form of cytochrome P_{450}; second, two forms of cytochrome P_{450} are present but both are equally affected by DDT, phenobarbital and benzpyrene. The latter appears more tenable since experiments now being conducted in our laboratory indicate that pretreatment with piperonyl butoxide causes a shift in the ethyl isocyanide equilibrium point in this particular strain of mice. This suggests that the potential for specific induction exists in this animal even through DDT and benzpyrene—normally specific inducers—appear to act in the same manner as phenobarbital.

Vesell[17] studied 11 strains of mice and found that five had significantly longer hexobarbital sleeping times. His results demonstrated that genetic factors are important in the response of different strains of mice. If the mechanism of induction is the result of a derepression process,[18] then DDT, phenobarbital and benzpyrene would be suspected to react in the same manner with the structural repressor.

Results from the present study and from other experiments[8–11, 15–17] indicate that caution should be used in making extrapolations of the effects of chemicals on different strains of an animal and different animals.

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