Biochemical Pharmacology, 1966, Vol. 15, pp. 137–144. Pergamon Press Ltd., Printed in Great Britain.

STUDIES OF INTERACTIONS OF 3,4-BENZPYRENE, 3-METHYLCHOLANTHRENE, CHLORDANE, AND METHYLTESTOSTERONE AS STIMULATORS OF HEPATIC MICROSOMAL ENZYME SYSTEMS IN THE RAT*

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(*Received* 13 *August* 1965; *accepted* 5 *October* 1965)

Abstract—3,4-Benzpyrene (BP), 3-methylcholanthrene (MC), and chlordane all stimulate the hepatic microsomal enzymes, benzpyrene hydroxylase, zoxazolamine hydroxylase, NADPH oxidase, and the amount of CO-binding pigment in adult male Long-Evans rats. Chlordane stimulates nitro reductase, NADPH oxidase, and CO-binding pigment to a greater extent than does BP or MC; nitro reductase is unaffected by MC or BP. BP and MC stimulate benzpyrene hydroxylase threefold; stimulation by chlordane is slight. Zoxazolamine hydroxylase is stimulated by all three compounds to about the same extent.

The stimulatory effects of BP or MC, and chlordane upon zoxazolamine hydroxylase and CO-binding pigment are additive when chlordane is given with either MC or BP to the same animal, indicating that chlordane may stimulate the activity of these enzymes by mechanisms different from BP or MC. Such additive effects are not seen with benzpyrene hydroxylase or NADPH oxidase activity, indicating that stimulation of these enzymes by the three compounds may be by similar mechanisms.

Chlordane and methyltestosterone stimulate hepatic microsomal aminopyrine N-demethylase activity of adult female Long-Evans rats. Additive stimulatory effects upon aminopyrine N-demethylase are not seen when chlordane and methyltestosterone are given together to the same animal, indicating that these two compounds may stimulate this drug-metabolizing pathway by similar mechanisms.

WIDE varieties of drugs and chemicals possess the ability to produce increases in the activities of hepatic microsomal drug-metabolizing enzyme systems. The long-acting barbiturates, chlorinated hydrocarbon insecticides, polycyclic aromatic hydrocarbons and certain anabolic steroids are among the most potent of these agents.

Previous reports have described the interactions of phenobarbital and chlordane,[1] phenobarbital and methyltestosterone,[2] phenobarbital and 3,4-benzpyrene,[2] and 3,4-benzpyrene and 3-methylcholanthrene.[2] Such studies seem to indicate that the long-acting barbiturates, polycyclic aromatic hydrocarbons, and anabolic steroids exert their stimulatory effect through different mechanisms, whereas chlordane and phenobarbital appear to act through similar mechanisms.

The present paper represents a further attempt to elucidate differences between these agents with regard to their ability to increase the activity of hepatic drug-metabolizing

* Supported by Grants GM 06034 and CA 05648 from the National Institutes of Health, Bethesda, Md.

enzymes. In addition, studies have also been carried out on the enzyme system in microsomes which oxidizes NADPH to NADP and on the amount of the microsomal carbon monoxide (CO)-binding pigment. It has been proposed that these systems are involved in the microsomal metabolism of foreign compounds.[3–5]

METHODS

Preparation of animals. For studies of the interaction of chlordane with 3,4-benzpyrene or 3-methylcholanthrene, male Long-Evans rats weighing 150–200 g were used. For studies of the interaction of chlordane and methyltestosterone, female Long-Evans rats weighing 150–200 g were used. All rats were kept in the animal house 4–7 days before beginning injections.

All compounds studied were dissolved in corn oil and injected intraperitoneally. 3,4-Benzpyrene (20 mg/kg) and 3-methylcholanthrene (20 mg/kg) were each administered 48 hr prior to sacrifice. Chlordane (100 mg/kg), as the γ isomer (98·9 $\pm$ 0·3% pure), was administered every day for 3 days, and animals were sacrificed on the fourth day. Methyltestosterone (20 mg/kg) was administered every other day for 14 days, and animals were sacrificed 24 hr after the last injection.

The above doses were found to give maximal stimulatory responses by dose–response studies which used half and double the above doses for each drug-metabolizing pathway studied. In no instance were the slopes of the regression lines significantly different from zero, nor did values of drug metabolism obtained for the three doses differ significantly from each other.

Preparation of tissue samples. Rats were killed by a blow on the head and their livers excised and placed on ice. The livers were homogenized in the cold with a Potter homogenizer having a plastic pestle. The resulting suspension contained 2 ml of cold 1·15% KCl for each gram of liver. The 9,000-*g* supernatant fraction, containing microsomal and soluble enzymes, was prepared from the homogenate by centrifugation at 9,000 *g* with a Lourdes model AX high-speed angle centrifuge (20 min) at 5°.

For assays of NADPH-oxidase and CO-binding pigment, microsomes were separated from the soluble fraction by centrifugation for 60 min at 78,000 *g* in a Spinco ultracentrifuge. Supernatant was discarded, the pellet resuspended in 1·15% KCl, and recentrifuged. This pellet was suspended in 0·1 M phosphate buffer, pH 7·4, so that the microsomes from 1 g liver were contained in 1 ml of suspension for the former assay, and microsomes from 1 g liver in 10 ml of suspension for the latter assay.

Assays in vitro. For determination of drug-metabolizing enzyme activity, 1 ml of hepatic 9000-*g* supernatant fraction was incubated in a Dubnoff shaking incubator for 1 hr at 37° with oxygen as the gaseous phase. For the reductive pathway, cell fractions were incubated under nitrogen for 1 hr at 37°. Final concentrations of cofactors added were: triphosphopyridine nucleotide (NADP) ($1{\cdot}1 \times 10^{-4}$ M), glucose-6-phosphate (5×10^{-3} M), nicotinamide (2×10^{-2} M), and $MgSO_4$ (5×10^{-3} M). All concentrations were supraoptimal. Final volume of all incubation mixtures was brought up to 5 ml with 0·1 M phosphate buffer, pH 7·4.

The pathways studied and substrate concentrations in μmoles per 5 ml incubate were: the N-dealkylation of aminopyrine (40 μmoles) to 4-aminoantipyrine, hydroxylation of benzpyrene (0·6 μmole) and zoxazolamine (3 μmoles), the reduction of the aromatic nitro group of *p*-nitrobenzoic acid (12 μmoles) to *p*-aminobenzoic acid.

Disappearance of the substrates benzpyrene and zoxazolamine were measured by the methods of Conney *et al.*[6] and Conney *et al.*[7], respectively, as modified by Juchau *et al.*[8] Appearance of the metabolites, 4-aminoantipyrine and *p*-aminobenzoic acid, was measured by the methods of Brodie and Axelrod[9] and Fouts and Brodie,[10] respectively.

The rate of oxidation of NADPH to NADP was determined by the method of Gillette *et al.*[11] as modified by Hart and Fouts.[1] The CO-binding pigment of Klingenberg was determined by the method of Klingenberg,[12] as modified by Hart and Fouts.[1]

All results are given in terms of milligrams of nitrogen (N). Total N was determined by a micro-Kjeldahl method as described by Todd *et al.*[13]

Experimental design. The design of this study was essentially the same as that used by Gillette[2] and Hart and Fouts.[1] When two drugs produce the same response, whether they do so by a similar mechanism or by different mechanisms may be elucidated by this design. If the mechanism is different, maximal doses of the two drugs should, when given together, produce a response significantly greater than either given alone. If the mechanism is the same, such addition of response should not occur.

Statistics. The statistical methods used are described by Steel and Torrie.[14] Analysis of variance and Duncan's new multiple range test were applied to the data. The level of significance chosen was $P < 0{\cdot}05$.

RESULTS

Effects of chlordane, 3-methylcholanthrene, and 3,4-benzpyrene on microsomal enzyme systems

For each of the five enzyme systems studied, 3,4-benzpyrene and 3-methylcholanthrene demonstrated a similar pattern (Table 1), supporting the work of Gillette. However, chlordane stimulation elicited a different pattern. Whereas 3,4-benzpyrene and 3-methylcholanthrene both greatly stimulated benzpyrene hydroxylase activity, chlordane stimulation was very slight. All three compounds stimulated zoxazolamine hydroxylase to about the same extent. 3,4-Benzpyrene and 3-methylcholanthrene had no effect upon nitro reductase activity, whereas chlordane stimulated activity fivefold. Chlordane also stimulated NADPH oxidase and CO-binding pigment to a greater extent than did 3,4-benzpyrene or 3-methylcholanthrene.

For zoxazolamine hydroxylase and CO-binding pigment, we were able to obtain significant addition of the stimulatory responses by maximal doses of either 3,4-benzpyrene or chlordane (Table 2) when both 3,4-benzpyrene and chlordane were given together, indicating that, for these two enzyme systems, chlordane and 3,4-benzpyrene may produce this response by different mechanisms. However, the responses of the enzyme systems benzyprene hydroxylase and NADPH oxidase to maximal doses of 3,4-benzpyrene or chlordane were no greater when both were given together than when either was given alone, indicating that chlordane and 3,4-benzpyrene may induce these enzyme systems by a similar mechanism. 3-Methylcholanthrene and chlordane (Table 3) also showed the same phenomena—that is, for zoxazolamine hydroxylase and CO-binding pigment, the responses to maximal doses of 3-methylcholanthrene and chlordane were additive when both were given together, while the stimulation of benzpyrene hydroxylase and NADPH oxidase by maximal doses of 3-methylcholanthrene and chlordane was not additive when both drugs were given together.

TABLE 1. STIMULATION OF HEPATIC MICROSOMAL METABOLISM BY CHLORDANE, 3,4-BENZPYRENE, AND 3-METHYLCHOLANTHRENE

Treatment	Dose	Benzpyrene hydroxylase* (μmole/mg N)‡	Zoxazolamine hydroxylase (μmole/mg N)‡	Nitro reductase (μmole/mg N)‡	NADPH oxidase (ΔO.D. at 340 mμ/min/mg N)§	CO-binding pigment (ΔO.D. at 450/mg N)§	N†
Corn oil	1 ml/kg	0·023	0·096	0·070	0·105	0·102	15
3,4-Benzpyrene	20 mg/kg	0·077	0·247	0·078	0·145	0·170	15
3-Methylcholanthrene	20 mg/kg	0·070	0·211	0·069	0·190	0·180	11
Chlordane	100 mg/kg	0·036	0·213	0·345	0·273	0·250	15
C.V.¶		24·3%	32·3%	21·4%	28·0%	31·7%	

* In each column, any values joined by a straight line are not significantly different ($P > 0.05$), while any values not joined by a straight line are significantly different ($P \leq 0.05$).
† The number at the right of each row indicates the number of animals used to determine each mean.
‡ Enzyme activity in μmoles substrate metabolized in 1 hr per mg of 9,000-*g* supernatant nitrogen.
§ Change in optical density at wavelength given, per mg of microsomal nitrogen.
¶ C.V. = coefficient of variability (all tables).

TABLE 2. STIMULATORY EFFECTS OF CHLORDANE AND 3,4-BENZPYRENE ON HEPATIC MICROSOMAL ENZYME ACTIVITY WHEN GIVEN ALONE OR IN COMBINATION

Treatment	Dose	Benzpyrene hydroxylase* (μmole/mg N)†	Zoxazolamine hydroxylase (μmole/mg N)†	NADPH oxidase (ΔO.D. at 340 mμ/min/mg N)‡	CO-binding pigment (ΔO.D. at 450/mg N)‡
Corn oil	1 ml/kg	0·026	0·104	0·119	0·096
3,4-Benzpyrene	20 mg/kg	0·088	0·244	0·169	0·160
Chlordane	100 mg/kg	0·036	0·215	0·270	0·223
Chlordane + 3,4-benzpyrene		0·075	0·326	0·235	0·276
C.V.		11·7%	24·2%	28·0%	25·0%
N§		8	8	8	8

* In each column, any values joined by a straight line are not significantly different ($P > 0·05$), while any values not joined by a straight line are significantly different ($P < 0·05$).

† Enzyme activity in μmoles substrate metabolized in 1 hr per mg of 9,000-*g* supernatant nitrogen.

‡ Change in O.D. at wavelength given, per mg of microsomal nitrogen.

§ Each value is the mean of that number of animals given at the bottom of the column.

TABLE 3. STIMULATORY EFFECTS OF CHLORDANE AND 3-METHYLCHOLANTHRENE ON HEPATIC MICROSOMAL ENZYME ACTIVITY WHEN GIVEN ALONE OR IN COMBINATION

Treatment	Dose	Benzpyrene hydroxylase* (μmole/mg N)†	Zoxazolamine hydroxylase (μmole/mg N)†	NADPH oxidase (ΔO.D. at 340 mμ/min/mg N)‡	CO-binding pigment (Δ O.D. at 450/mg N)‡
Corn oil	1 ml/kg	0·026	0·104	0·119	0·096
3-Methylcholanthrene	20 mg/kg	0·077	0·229	0·218	0·178
Chlordane	100 mg/kg	0·036	0·215	0·270	0·223
Chlordane + 3-methylcholanthrene		0·079	0·368	0·277	0·270
C.V.		11·7%	24·2%	31·0%	28·2%
N§		8	8	8	8

* In each column, any values joined by a straight line are not significantly different ($P > 0·05$), while any values not joined by a straight line are significantly different ($P < 0·05$). In the column headed "Benzpyrene hydroxylase" the values 0·077 and 0·079 are not significantly different from each other, but are both significantly different from all other values.

† Enzyme activity in μmoles substrate metabolized in 1 hr per mg of 9,000-*g* supernatant nitrogen.

‡ Change in O.D. at wavelength given, per mg of microsomal nitrogen.

§Each value is the mean of that number of animals given at the bottom of the column.

Effects of methyltestosterone and chlordane on the rate of aminopyrine metabolism

Hart and Fouts[1] showed that phenobarbital and chlordane when given together did not significantly add to the effects of either given alone upon the rate of aminopyrine metabolism. Gillette[2] demonstrated that stimulation of the rate of monomethyl-4-aminoantipyrine metabolism by maximal doses of methyltestosterone and phenobarbital was significantly greater when both were given together than when each was given individually. On the basis of this work, we expected that the effects of maximal doses of methyltestosterone and chlordane on aminopyrine metabolism would be additive when both were given together. However, we did not find this to be so (Table 4). Stimulation of aminopyrine metabolism by maximal doses of methyltestosterone and chlordane together was no greater than stimulation by chlordane alone.

TABLE 4. STIMULATORY EFFECTS OF CHLORDANE AND METHYLTESTOSTERONE ON THE RATE OF AMINOPYRINE METABOLISM BY LIVER MICROSOMES FROM FEMALE RATS

Treatment	Dose	4-Aminoantipyrine formed (μmole/mg N)*
Corn oil	1 ml/kg	0·024
Methyltestosterone	20 mg/kg	0·047
Chlordane	100 mg/kg	0·133
Chlordane + methyltestosterone		0·116
C.V.		37·5%
N†		4

* Any values joined by a straight line are not significantly different ($P > 0·05$), while any values not joined by a straight line are significantly different ($P < 0·05$). Enzyme activity in μmoles per mg of 9,000-*g* supernatant nitrogen.

† Each value is the mean of four determinations.

DISCUSSION

One approach to the study of the mechanism of action of increased hepatic microsomal enzyme activity induced by certain drugs and chemicals utilizes observation of their interactions on systems which are induced by each agent. The results indicate that chlordane, 3,4-benzpyrene, and 3-methylcholanthrene all produce significant increases in the activities of benzpyrene hydroxylase, zoxazolamine hydroxylase, NADPH oxidase, and in the amount of CO-binding pigment. Chlordane and methyltestosterone are both capable of causing significant increases in the metabolism of aminopyrine *in vitro*.

In most cases, 3,4-benzpyrene and 3-methylcholanthrene behaved similarly with regard to enzyme stimulations. However, the following differences were observed. (1) 3-Methylcholanthrene produced a significantly greater increase than did 3,4-benzpyrene in NADPH oxidase activity. (2) The increase in activity of benzpyrene hydroxylase produced by the administration of 3,4-benzpyrene was significantly decreased when administered simultaneously with chlordane; such a decrease was not observed when chlordane and 3-methylcholanthrene were administered together. (3) The increase in amount of CO-binding pigment produced by chlordane was significantly enhanced by concomitant administration of 3,4-benzpyrene but not by concurrent administration of 3-methylcholanthrene (the per cent increases were similar

however). Thus it appears that even though both 3,4-benzpyrene and 3-methylcholanthrene seem to stimulate microsomal systems through similar mechanisms (as reported by Conney *et al.*[15] and Gillette[2]), some differences were observed.

Chlordane and the polycyclic aromatic hydrocarbons exhibit many dissimilar effects on hepatic microsomal systems, as demonstrated by this study and by other investigations. The differences are evident not only in the wider variety of enzymic pathways which chlordane is capable of stimulating, but also in the extent to which they are affected. Cytological differences in effects on liver have also been observed by Fouts and Rogers.[16] These latter studies demonstrated a marked proliferation of smooth endoplasmic reticulum in livers of animals which had been pretreated with chlordane. Such a pronounced membrane proliferation did not occur in livers of animals pretreated with 3,4-benzpyrene or 3-methylcholanthrene. Thus, the present study provides further evidence to justify separate classifications of the polycyclic aromatic hydrocarbons and chlorinated hydrocarbon insecticides as stimulators of hepatic microsomal enzyme systems.

Although phenobarbital and chlordane exhibit many similarities as enzyme stimulators, we observed that simultaneous administration of chlordane and methyltestosterone did not produce additive stimulatory effects on aminopyrine N-demethylation. Gillette,[2] as mentioned previously, noted additive effects of phenobarbital and methyltestosterone on this enzymic pathway. The results obtained in our study may indicate slightly different effects of phenobarbital and chlordane similar to those mentioned concerning 3,4-benzpyrene and 3-methylcholanthrene. Hart and Fouts[1] were unable to reverse ethionine-induced blockade of chlordane stimulation with methionine, whereas reversal was obtained in the case of ethionine blockade of phenobarbital stimulation. Such observations are not surprising in view of the probable complexity of the induction process. Each enzyme-stimulating agent may be capable of acting at several different points in the overall process by which enzyme activity is affected.

REFERENCES

1. L. G. HART and J. R. FOUTS, *Biochem. Pharmac.* **14**, 263 (1965).
2. J. R. GILLETTE, *Advances in Enzyme Regulation*, G. WEBER, Ed., vol. 1, p. 215, Pergamon Press, London (1963).
3. A. H. CONNEY and J. J. BURNS, *ibid.*, p. 206.
4. R. REICHERT and H. REMMER, *Naunyn-Schmiedeberg's Arch exp. Path. Pharmak.* **247**, 374 (1964).
5. S. ORRENIUS and L. ERNSTER, *Biochem. biophys. Res. Commun.* **16**, 60 (1964).
6. A. H. CONNEY, E. C. MILLER and J. A. MILLER, *J. biol. Chem.* **228**, 753 (1957).
7. A. H. CONNEY, J. R. GILLETTE, J. K. INSCOE, E. G. TRAMS and H. S. POSNER, *Science* **130**, 1478 (1959).
8. M. R. JUCHAU, R. L. CRAM, G. L. PLAA and J. R. FOUTS, *Biochem. Pharmac.* **14**, 473 (1965).
9. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **99**, 171 (1950).
10. J. R. FOUTS and B. B. BRODIE, *J. Pharmac. exp. Ther.* **119**, 197 (1957).
11. J. R. GILLETTE, B. B. BRODIE and B. N. LADU, *J. Pharmac. exp. Ther.* **119**, 532 (1957).
12. M. KLINGENBERG, *Archs. Biochem.* **75**, 376 (1958).
13. J. TODD, A. SANFORM and B. WELL, *Clinical Diagnosis by Laboratory Methods*, 12th ed. Saunders, Philadelphia (1963).
14. R. G. D. STEEL and J. H. TORRIE, *Principles and Procedures of Statistics*, McGraw-Hill, New York (1960).
15. A. H. CONNEY, C. DAVISON, R. GASTEL and J. J. BURNS, *J. Pharmac. exp. Ther.* **130**, 1 (1960).
16. J. R. FOUTS and L. A. ROGERS, *J. Pharmac. exp. Ther.* **147**, 112 (1965).