Effects of Metyrapone on Liver Microsomal Drug Oxidations

KENNETH C. LEIBMAN

Department of Pharmacology and Therapeutics,
University of Florida College of Medicine, Gainesville, Florida 32601

(Received September 11, 1968)

SUMMARY

Metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propanone], an inhibitor of adrenal steroid hydroxylation, has been shown to affect a number of liver microsomal drug-oxidizing enzyme systems. The metabolism of hexobarbital and that of aminopyrine are inhibited *in vitro* by this drug in microsomal preparations from control and phenobarbital-treated rats. Hexobarbital sleeping time is prolonged by metyrapone in both groups of rats. Demethylation of morphine and hydroxylation of tyramine are inhibited to a lesser extent, and NADPH oxidase is little affected. In contrast, the effect of metyrapone on the formation of phenolic metabolites from acetanilide follows a biphasic relationship with respect to metyrapone concentration: inhibition occurs only at very high concentrations, and the reaction is greatly enhanced at lower concentrations with both control and phenobarbital-induced preparations. Trichloroethylene oxidation is also enhanced by metyrapone in both preparations, but the enhancement in phenobarbital-induced preparations is superimposed on an inhibitory effect. Studies with the latter pathway show that both effects are independent of the NADPH-generating system, and are exerted in a reversible manner.

INTRODUCTION

Metyrapone [SU-4885, 2-methyl-1,2-bis (3-pyridyl)-1-propanone] is a potent inhibitor of adrenal steroid 11β-hydroxylase (1), and also inhibits to various extents hydroxylation at other points on steroid molecules in the adrenal gland (2). The adrenal steroid 11β-hydroxylase system is dependent on NADPH and oxygen, and contains cytochrome P-450 as its terminal oxidase (3). In these respects, it is remarkably similar to the enzyme systems of liver microsomes which mediate the oxidation of a large group of xenobiotic chemicals. Kuntzman, Conney *et al.* (4, 5) studied steroid hydroxylation in liver microsomes and suggested that steroid hormones are normal substrates for microsomal drug-metabolizing enzymes.

It thus became of interest to study the effect of metyrapone on various drug oxidations in liver microsomes. Such studies showed that metyrapone inhibited the oxidative metabolism of hexobarbital and of aminopyrine at relatively low concentrations, and that of acetanilide at considerably higher concentrations (6). Similar results have independently been obtained by Netter *et al.* (7). Extensions of these experiments are reported in detail in this paper.

This research was supported in part by United States Public Health Service Grants OH-00124 and OH-00251.

METHODS

The enzyme preparation was reconstituted as needed from lyophilized 9000 $\times$ g supernatant fractions, prepared as previously described (8) from livers of male Holtzman rats weighing 125–150 g. Isolated microsome fractions were also obtained from these preparations (9). Treatment with sodium phenobarbital consisted of intraperitoneal injections of 75 mg/kg

daily for 4 days, the last injection occurring about 20 hr before the animal was killed.

Enzyme assays were performed by published methods with slight modifications; thus Tris-HCl buffer was used throughout. With this exception, the incubation conditions for studying *N*-demethylation of aminopyrine were those of La Du *et al.* (10). The demethylated product, 4-aminoantipyrine, was measured by the method of Brodie and Axelrod (11), modified by increasing the volume of isoamyl alcohol to allow the use of larger cuvettes. Hexobarbital metabolism was assayed by the procedure described by Cooper and Brodie (12). Incubations with morphine were carried out in the system of Leadbeater and Davies (13), and the formaldehyde produced by oxidative demethylation was measured as described by Cochin and Axelrod (14). Conditions for study of tyramine hydroxylation were those of Lemberger *et al.* (15), and the dopamine formed was measured by the procedure of Anton and Sayre (16). The method of Gillette *et al.* (17) was used for assay of NADPH oxidase activity. Acetanilide hydroxylation was studied as described by Krisch and Staudinger (18), with the important exception that the incubation mixture was extracted without prior acidification in order to avoid artifactual results (19). Oxidation of trichloroethylene to chloral hydrate was measured by a method developed in this laboratory (9). Metyrapone base was added to reaction mixtures as a freshly prepared aqueous solution.

Sleeping times were measured from the loss to the regaining of righting reflex, using male Holtzman rats weighing 75–90 g.

RESULTS

Aminopyrine demethylation. The effect of metyrapone on the oxidative *N*-demethylation of aminopyrine is shown in Fig. 1. In preparations from both phenobarbital-treated and control rats, metyrapone caused increasing inhibition of the formation of 4-aminoantipyrine when it was added in increasing concentrations to the assay mixtures *in vitro*. The concentration of metyrapone causing 50% inhibition of demethylase activity was the same with both preparations, 5×10^{-5} M.

Hexobarbital metabolism. Similar experiments on the metabolism of hexobarbital

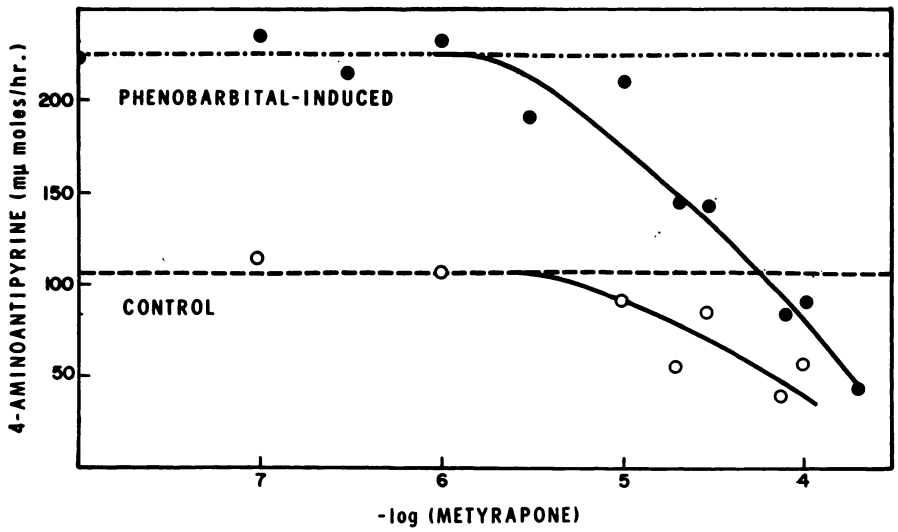

FIG. 1. *Inhibition in vitro of N-demethylation of aminopyrine by metyrapone*

Substrate concentration was 2×10^{-3} M; glucose 6-phosphate, 5×10^{-3} M. ○, Lyophilized 9000 × *g* supernatant fraction from 790 mg of liver from control rats; ●, similar fraction from 280 mg of liver from phenobarbital-treated rats. Broken lines indicate activity of the two preparations in the absence of metyrapone.

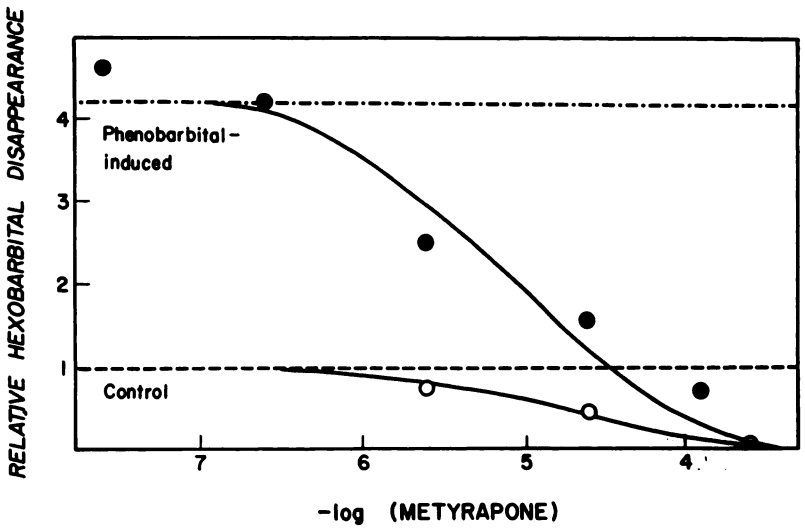

FIG. 2. *Inhibition in vitro of hexobarbital metabolism by metyrapone*

Substrate concentration was 2.5×10^{-4} M. Glucose 6-phosphate, 6.25×10^{-3} M. ○, Lyophilized 9000 × *g* supernatant fraction from 640 mg of liver from control rats; ●, similar fraction from 115 mg of liver from phenobarbital-treated rats. Broken lines indicate activity of the two preparations in the absence of metyrapone. The basal rate of hexobarbital metabolism for the control case was 260 mμmoles/hr.

in the preparations from induced and control rat livers are shown in Fig. 2. Metyrapone appears to be a somewhat more potent inhibitor of hexobarbital than of aminopyrine oxidation. Essentially complete inhibition of the disappearance of hexobarbital from reaction mixtures containing either preparation was caused by 2.5×10^{-4} M metyrapone. This assay, which depends on the measurement of substrate disappearance, is inherently less precise than that for aminopyrine demethylation, in which product formation is determined. It is therefore not possible to pinpoint the concentration that caused 50% inhibition in the presence of either preparation, nor can it be stated with certainty whether this concentration was the same or different in the two cases. However, metyrapone reduced the rate of hexobarbital disappearance from both systems *in vitro* to one-half of control values at a concentration near 10^{-5} M.

Table 1 shows the sleeping times after a test dose of hexobarbital was administered to control and phenobarbital-treated rats, some of each of which had received an injection of metyrapone 30 min before challenge with hexobarbital. In rats which had received no prior treatment with phenobarbital, metyrapone caused a remarkable increase in the hexobarbital sleeping time. The mean sleeping time of the animals that had received metyrapone was about 4 times that of the controls, and one of the metyrapone-treated rats slept for over 9 hr. As has been observed many times, the hexobarbital sleeping time was greatly

TABLE 1

Effect of metyrapone on hexobarbital sleeping time in rats

Prior treatment	Metyrapone[a]	*N*	Sleeping time[b]	*p*[c]
			min ± *SE*	
None	0	6	70 ± 5	<0.005
	+	7	309 ± 57	
Phenobarbital	0	6	10 ± 1	<0.01
	+	7	26 ± 5	

[a] The dose of metyrapone was 60 mg/kg, given intraperitoneally 30 min before hexobarbital.

[b] Response to a 100 mg/kg intraperitoneal dose of hexobarbital.

[c] Probability that observed differences were due to chance, calculated from Student's *t*-test.

TABLE 2

Effect of metyrapone on various microsomal oxidations

Substrate concentrations were: morphine sulfate, 5×10^{-4} M; tyramine hydrochloride, 7×10^{-3} M; NADPH, 8×10^{-4} M. Lyophilized 9000 × *g* supernatant fraction from 500 mg of liver (wet weight) was used for assay of morphine demethylation and tyramine hydroxylation. Microsomes from 400 mg of liver (wet weight) were used for the NADPH oxidase assay. All preparations were from phenobarbital-treated animals.

Metyrapone	Morphine demethylation	Tyramine hydroxylation	NADPH oxidase
mM	*μmole HCHO/hr*	*mμmoles dopamine/hr*	$\Delta A_{340}/min$
0	0.72, 0.74	37, 37	0.082, 0.081
1.0	0.38, 0.42	19, 19	0.064, 0.061
Inhibition	47%	48%	23%

shortened by prior treatment of the rats with phenobarbital. Here, too, metyrapone caused an increase in response to the test dose of hexobarbital, although the proportional increase (2.5-fold) was not as great as in rats in which the liver microsomal enzymes had not been induced with phenobarbital.

Other enzymes inhibited by metyrapone. The effect of metyrapone on a number of other microsomal oxidations is shown in Table 2. Inhibition of the *N*-demethylation of morphine reached 50% only at a metyrapone concentration of about 10^{-3} M. The same was true for the hydroxylation of tyramine to dopamine in the induced microsomal system. NADPH oxidase activity was inhibited to a lesser extent by 10^{-3} M metyrapone.

Acetanilide hydroxylation. When the effect of metyrapone on the hydroxylation of acetanilide in the two rat liver microsomal preparations was studied, the results displayed in Fig. 3 were obtained. No inhibition of phenol formation could be seen at metyrapone concentrations below about 10^{-2} M. Instead, in preparations from both control and phenobarbital-treated rats, enhancement of the reaction took place, with a doubling of the extent of hydroxylation

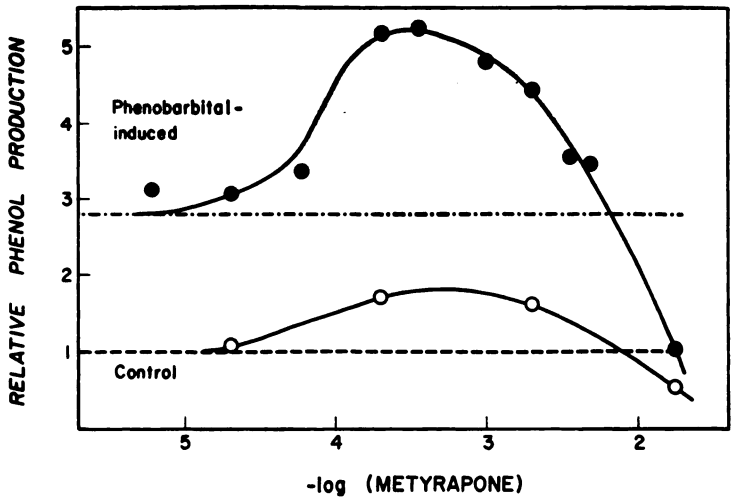

FIG. 3. *Effects of metyrapone in vitro on hydroxylation of acetanilide*

Substrate concentration was 4×10^{-3} M. ○, Lyophilized 9000 × *g* supernatant fraction from 400 mg of liver from control rats; ●, similar fraction from 400 mg of liver from phenobarbital-treated rats. Broken lines indicate activity of the two preparations in the absence of metyrapone. Phenol production is given in arbitrary units based on spectrophotometer readings in a standard procedure.

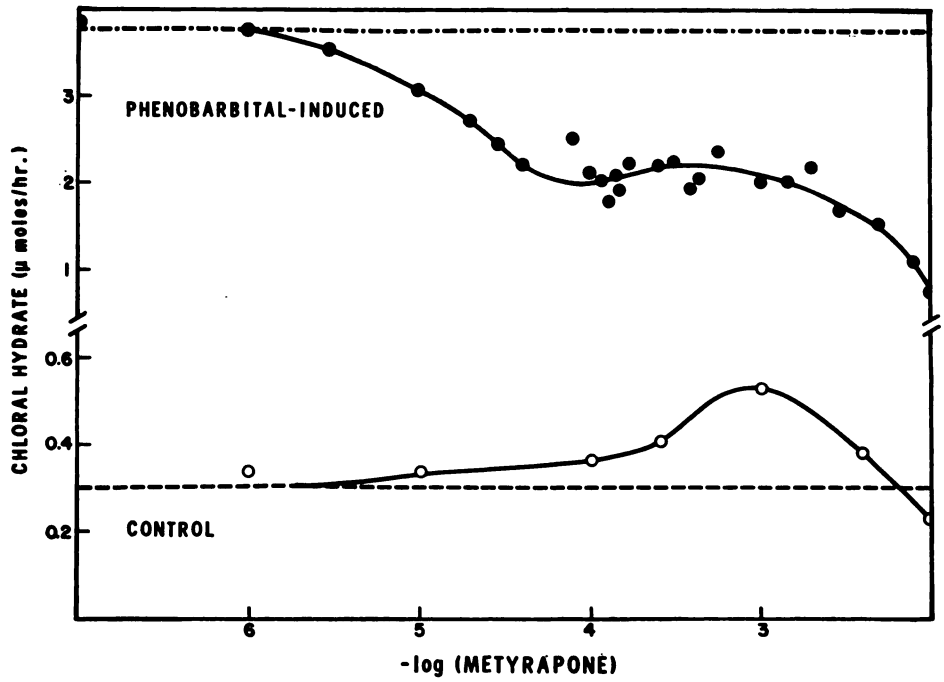

FIG. 4. *Effects of metyrapone in vitro on trichloroethylene oxidation*

Substrate concentration was 2% of the total volume (see ref. 9). ○, Lyophilized 9000 × *g* supernatant fraction from 400 mg of liver from control rats; ●, similar fraction from 400 mg of liver from phenobarbital-treated rats. Experiments with the induced preparation contained 4×10^{-3} M glucose 6-phosphate. Broken lines indicate activity of the two preparations in the absence of metyrapone.

occurring at about 5×10^{-4} M metyrapone in each case. Inhibition was seen in both preparations when the metyrapone concentration was raised to 2×10^{-2} M. This hydroxylation, in contrast to other microsomal oxidations (9), is not affected by glucose 6-phosphate; preliminary experiments showed that metyrapone caused similar effects in the presence or absence of glucose 6-phosphate.

Trichloroethylene oxidation. Still another pattern of inhibition was encountered when metyrapone was added to reaction mixtures in which the oxidation of trichloroethylene to chloral hydrate was taking place. Figure 4 shows that in enzyme preparations from control rats, little or no change in trichloroethylene oxidation was caused by metyrapone at concentrations below 10^{-4} M. As the concentration was raised, enhancement of the oxidative reaction was observed, and a maximal rate increase of 67% occurred at 10^{-3} M metyrapone. Chloral hydrate formation then declined, and inhibition occurred when the metyrapone concentration was 10^{-2} M.

In preparations from livers of rats that had been treated with phenobarbital, inhibition of trichloroethylene oxidation was observed when metyrapone was present in concentrations greater than 10^{-6} M At higher drug concentrations, however, the typical sigmoid inhibition curve was not generated. Rather, at metyrapone concentrations above 10^{-4} M, the rate of oxidation increased, and the inhibition curve for this preparation tended to parallel that of the "noninduced" preparation. Despite this reversal in behavior, the oxidation rates in the presence of metyrapone at concentrations between 10^{-4} and 10^{-2} M never reached that observed in the absence of drug. Glucose 6-phosphate slightly inhibits trichloroethylene oxidation in noninduced preparations, but greatly assists this reaction in phenobarbital-induced enzyme

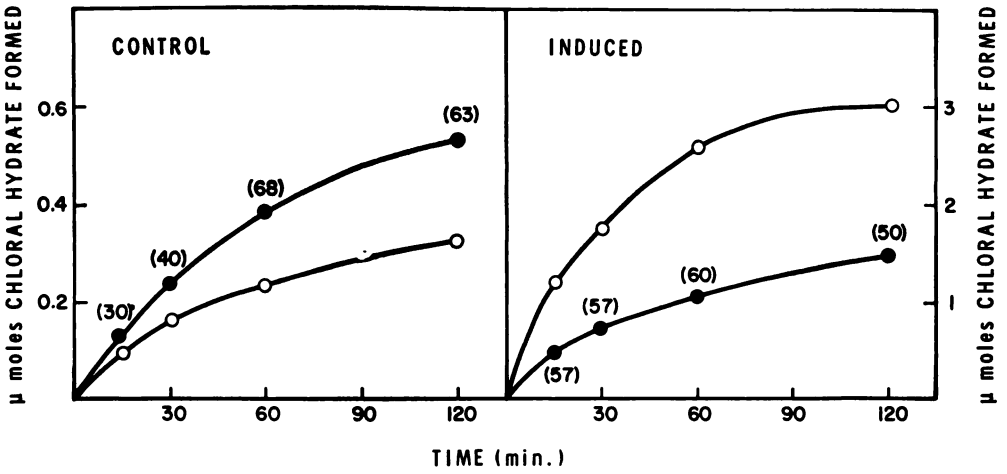

FIG. 5. *Time course of metyrapone effects on trichloroethylene oxidation in vitro*

Conditions were the same as in Fig. 4. Experiments were performed in absence (○) or presence (●) of metyrapone, 1×10^{-3} M with noninduced enzyme and 3×10^{-3} M with induced enzyme. Percentage enhancement or inhibition is shown in parentheses.

preparations (9). For this reason, glucose 6-phosphate was omitted here in control experiments, but included when the induced enzyme preparations were used. Preliminary experiments showed that induced preparations exhibited qualitatively similar behavior toward metyrapone in the presence or absence of glucose 6-phosphate.

The time courses of both these effects are charted in Fig. 5. In preparations derived from control rats, shown at the left, metyrapone caused enhancement of trichloroethylene oxidation at the earliest time studied. The degree of enhancement tended to increase as the incubation time was lengthened. In the preparations from phenobarbital-treated animals, inhibition by metyrapone was evident from the start of the reaction and remained approximately constant throughout the assay, as shown in the right-hand diagram.

Ackermann-Potter plots showing the influence of enzyme concentration on the metyrapone effects are presented in Fig. 6. In the case of the induced enzyme preparation, the classical picture (20) of reversible inhibition of trichloroethylene by metyrapone was displayed. With the control preparation, an analogous pair of lines was obtained, showing a constant degree of enhancement with changing enzyme concentration. This might be described as "reversible enhancement."

When the oxidation of trichloroethylene was measured in isolated microsomal fractions of rat liver with added NADPH but in the absence of an NADPH-generating system, differential effects of metyrapone were again observed in preparations from control rats and from those which had received prior treatment with phenobarbital. Table 3 shows that 10^{-3} M metyrapone caused a 36% enhancement of chloral hydrate formation in the presence of control microsomes, while with phenobarbital-induced microsomes the reaction was inhibited by 42%.

TABLE 3
Effects of metyrapone on trichloroethylene oxidation in isolated microsomes

Preparation	Metyrapone	Chloral hydrate formed	Change
	mM	*μmole/hr*	%
Noninduced	0	0.19	+36
	1	0.26	
Induced	0	0.91	−42
	3	0.53	

DISCUSSION

A number of drug-metabolizing, mixed-function oxidase systems of rat liver microsomes have been shown to be inhibited by metyrapone both in control and in phenobarbital-induced preparations (6). In one of these cases, that of the *N*-demethylation of aminopyrine, Netter *et al.* (7) have recently reported inhibition by metyrapone in preparations from phenobarbital-treated mice; thus some comparison between the two species is possible. In the presence of either control or induced rat liver preparations, in which the substrate concentration was 2×10^{-3} M, 50% inhibition was observed when the metyrapone concentration was 5×10^{-5} M. In the induced mouse liver preparation, with 1×10^{-3} M aminopyrine, the I_{50} of metyrapone was reported to be an order of magnitude higher, i.e., 5×10^{-4} M (7).

Another substrate studied in both laboratories was acetanilide. Although Netter *et al.* (7) showed inhibition curves for other metabolic reactions, they gave data only for one metyrapone concentration (2×10^{-3} M) in the case of acetanilide hydroxylation. With a substrate concentration of 1.85×10^{-3} M, slightly less than 50% inhibition occurred in their experiment with phenobarbital-induced mouse liver $8000 \times g$ supernatant fraction. In the present investigation with rat liver preparations, in which the substrate concentration was 4×10^{-3} M, a significant enhancement of hydroxylation occurred at 2×10^{-3} M metyrapone, although this concentration was higher than optimal. Inhibition of phenol production was observed in the rat liver preparations when the metyrapone concentration was an order of magnitude higher.

Of the oxidative reactions studied, hexobarbital metabolism was the most profoundly inhibited by metyrapone. Since hexobarbital side chain hydroxylation rapidly leads to the formation of an inactive metabolite, factors affecting this process cause significant changes in duration of the pharmacological action of the barbiturate. This was found to be the case; rats that had received metyrapone 30 min before challenge with hexobarbital slept for appreciably longer periods than controls. This was true for both phenobarbital-treated and untreated animals, although the proportional effect was greater in the latter group. At this relatively low dose of

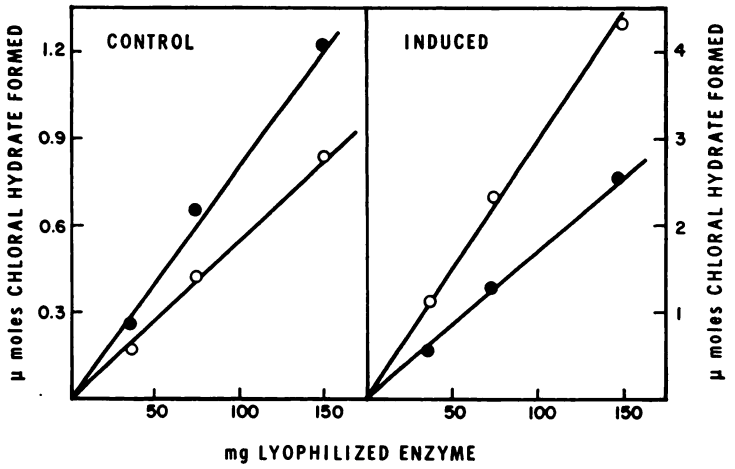

FIG. 6. *Metyrapone effects on trichloroethylene oxidation in vitro at different enzyme concentrations*

Conditions and symbols were the same as in Fig. 5, but with varying amounts of enzyme preparations. Additions of buffer were varied to compensate for Tris buffer introduced with the lyophilized enzyme. In these preparations, 1 mg of lyophilized powder was derived from 5.3 and 5.6 mg (wet weight) of control and induced livers, respectively. Incubation time was 1 hr.

metyrapone, no sedative effects were noted in the absence of hexobarbital. Since the time between the administration of metyrapone and that of hexobarbital was quite short, it is unlikely that the effects seen were mediated via the adrenal; they may therefore be ascribed to the direct action of metyrapone on the hexobarbital-oxidizing enzyme system.

NADPH oxidase activity, measured in the absence of other exogenous substrates, was affected very little by 10^{-3} M metyrapone. Netter *et al.* found no effect of this concentration of drug on NADPH oxidation measured during the simultaneous oxidation of *p*-nitroanisole (7). In addition, metyrapone has been shown to have no effect on the production of NADPH in the glucose 6-phosphate dehydrogenase reaction (7, 21). Further evidence that the coenzyme-reducing system has no influence on the effects of metyrapone is provided by the present study, in which isolated microsomal fractions were used. Enhancement of trichloroethylene oxidation occurred in the presence of control microsomes, whereas with induced microsomes, inhibition was observed. These effects paralleled those seen in experiments in which $9000 \times g$ supernatant fractions were employed as the enzyme source. Both the inhibitory and enhancing effects of metyrapone are therefore independent of the NADPH-generating system. They are apparently also not mediated through any interaction with the NADPH-cytochrome *c* reductase, which is thought to constitute the first step of the microsomal electron transport chain involved in the mixed-function oxidation of xenobiotic chemicals (22).

It would thus appear that the locus of action of metyrapone is at a later step of the microsomal electron transport chain, very likely at cytochrome P-450, the terminal oxidase-oxygenase of this chain (23). Recently Schenkman *et al.* (24) studied spectral changes resulting from the interaction of substrates of mixed-function oxidation with the microsomal cytochrome. They grouped substrates into two classes, depending on which of two greatly different spectral patterns due to such interaction were obtained. They suggested that substrates of the two types interact with different microsomal hemoproteins, or with different sites on a single species of cytochrome P-450. It is interesting that the two substrates whose oxidations were the most extensively inhibited by metyrapone, hexobarbital and aminopyrine, display type I spectral changes whereas acetanilide, whose oxidation was enhanced by metyrapone, falls in one of the subclasses of the type II spectral change. Spectral effects of the other substrates used here have not been reported. It is possible that metyrapone interacts with different substrate-binding sites in different ways, or that metyrapone reacts at a single locus in such a way as to cause different effects at different sites of substrate interaction.

The biphasic action of metyrapone on phenol production from acetanilide is remarkably similar to the effect of ethyl isocyanide on aniline hydroxylation, reported by Imai and Sato (25). These workers attributed the stimulatory effect of ethyl isocyanide to an increase in rate of reduction of oxidized cytochrome P-450 by NADPH, and the inhibitory action to a competition between ethyl isocyanide and oxygen for the heme of reduced cytochrome P-450. Aniline, like acetanilide, is a type II substrate (24). Imai and Sato found, however, that oxidative demethylation of the type I substrate aminopyrine was also affected in a dual manner by ethyl isocyanide. No stimulation of aminopyrine demethylation by metyrapone has been observed in the present work with preparations from either control or phenobarbital-treated animals.

ACKNOWLEDGMENTS

I thank Dr. J. J. Chart of the Ciba Pharmaceutical Company for generous supplies of metyrapone, Dr. A. H. Anton and Mr. D. F. Sayre for performing the determinations of dopamine, Dr. Betty Vogh for some of the data of Fig. 4, and Miss Elsa Ortiz for her excellent technical assistance.

REFERENCES

1. G. W. Liddle, D. Island, E. M. Lance and A. P. Harris, *J. Clin. Endocrinol. Metab.* **18**, 906 (1958).
2. N. P. Sanzari and F. G. Peron, *Steroids* **8**, 929 (1966).
3. D. Y. Cooper, S. Narasimhulu, A. Slade, W. Raich, O. Foroff and O. Rosenthal, *Life Sci.* **4**, 2109 (1965).
4. R. Kuntzman, M. Jacobson, K. Schneidman and A. H. Conney, *J. Pharmacol. Exp. Ther.* **146**, 280 (1964).
5. R. Kuntzman, D. Lawrence and A. H. Conney, *Mol. Pharmacol.* **1**, 163 (1965).
6. K. C. Leibman, *Fed. Proc.* **25**, 417 (1966).
7. K. J. Netter, S. Jenner and K. Kajuschke, *Arch. Pharmakol. Exp. Pathol. (Naunyn-Schmiedebergs)* **259**, 1 (1967).
8. K. C. Leibman, *Mol. Pharmacol.* **1**, 239 (1965).
9. K. C. Leibman and W. J. McAllister, Jr., *J. Pharmacol. Exp. Ther.* **157**, 574 (1967).
10. B. N. La Du, L. Gaudette, N. Trousof and B. B. Brodie, *J. Biol. Chem.* **214**, 741 (1955).
11. B. B. Brodie and J. Axelrod, *J. Pharmacol. Exp. Ther.* **99**, 171 (1950).
12. J. R. Cooper and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **114**, 409 (1955).
13. L. Leadbeater and D. R. Davies, *Biochem. Pharmacol.* **13**, 1569 (1964).
14. J. Cochin and J. Axelrod, *J. Pharmacol. Exp. Ther.* **125**, 105 (1959).
15. L. Lemberger, R. Kuntzman, A. H. Conney and J. J. Burns, *J. Pharmacol. Exp. Ther.* **150**, 292 (1965).
16. A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.* **145**, 326 (1964).
17. J. R. Gillette, B. B. Brodie and B. N. La Du, *J. Pharmacol. Exp. Ther.* **119**, 532 (1957).
18. K. Krisch and H. Staudinger, *Biochem. Z.* **334**, 312 (1961).
19. K. Krisch, H. Staudinger and V. Ullrich, *Life Sci.* **3**, 97 (1964).
20. W. W. Ackermann and V. R. Potter, *Proc. Soc. Exp. Biol. Med.* **72**, 1 (1949).
21. O. V. Dominguez and L. T. Samuels, *Endocrinology* **73**, 304 (1963).
22. A. H. Phillips and R. G. Langdon, *J. Biol. Chem.* **237**, 2652 (1962).
23. T. Omura, R. Sato, D. Y. Cooper, O. Rosenthal and R. W. Estabrook, *Fed. Proc.* **24**, 1181 (1965).
24. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Mol. Pharmacol.* **3**, 113 (1967).
25. Y. Imai and R. Sato, *J. Biochem. (Tokyo)* **63**, 380 (1968).