Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Drug Metabolism and Hepatic Microsomes of Rats and Mice

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Introduction

It has been reported (*1*) that one effect of a single, intra-peritoneal dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) is to cause a reduction in the duration of action of the drug 2-amino-5-chlorobenzoxazole (zoxazolamine) in the rat. This effect is maximal with doses of dioxin above 100 μg/kg and significant even at 5 μg/kg. Our own work showed that oral dosage had a similar effect but also that the duration of action of hexobarbitone was considerably prolonged (*2*). The results described here extend these observations.

Materials and Methods

Animals

Rats of the albino Porton strain bred in these Laboratories and weighing 180–200 g (male 6–8 weeks and female 7–10 weeks old) had free access to water and diet 41B unless otherwise indicated.

Mice of the C57BL/6 and DBA/2 strains were bred in these laboratories and kept on Sterolit bedding (Mineral & Chemical Corporation of America, Menlo Park, New Jersey, U.S.A.) for at least 3 weeks before use. They had free access to diet and water.

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Dosages

Dioxin, prepared as described elsewhere (*3*), was administered as a solution (100 μg/ml) in Arachis oil. Control animals received an equivalent volume of oil. Zoxazolamine (McNeil Laboratories, Inc.) was dissolved in 1*N* HCl (1.2 ml/100 mg), diluted with 0.9% NaCl to 10 mg/ml and administered IP at 100 mg/kg. 5-Cyclo-hex-1′-enyl-1,5-dimethylbarbituric acid (hexobarbitone) sodium (May & Baker Ltd.) was dissolved in water at 50 or 25 mg/ml and administered IP at 150 mg/kg (male rats), 75 mg/kg (female rats), 100 mg/kg (male and female mice). *dl*-Ethionine (Koch-Light) was dissolved in water (20 mg/ml) and administered as indicated in the text.

Microsomal Preparations

Liver microsomes were isolated either as described by Bond and De Matteis (*4*), except that the microsomes were sedimented at 105,000*g*, or by the calcium/sucrose method (*5*) adapted to the extent of sedimenting the calcium-treated microsomes at 1900*g* and washing them once in a 0.0125*M* sucrose solution containing 8*mM* $CaCl_2$. The final preparations were suspended in 0.1*M* phosphate buffer (pH 7.4) containing 1*mM* Na_2-EDTA (1.4 –7.0 mg protein/ml). Protein was estimated by the biuret method (*6*) and cytochrome P-450 (or P-448) by using an

extinction coefficient of 91/*mM*–cm (*7*) for the absorbancy change between 450 (448) and 490 nm of the CO difference spectrum of a $Na_2S_2O_4$-reduced suspension.

Assays

Plasma barbiturate levels following hexobarbitone administration were measured by the method of Chromý and Babjuk (*8*).

Hexobarbitone oxidase was measured *in vitro* by the modified (*9*) method of Brodie et al. (*10*).

Zoxazolamine hydroxylase was measured by the methods of Burns and his colleagues (*11, 12*) with minor modifications.

Difference Spectra

Difference spectra were recorded on a Unicam SP 1800 spectrophotometer with oxidized microsomal suspensions (2.7 ml, 1.4–2.2 mg protein /ml) in each 1-cm cuvet and the addition of either aniline (5–200 μl, 0.22-*M* in 0.1*M* phosphate buffer, pH 7.4) or hexobarbitone sodium (5-100 μl, 0.054*M* in water) to the sample cuvet and of an equal volume of the appropriate solvent to the reference cuvet. Pyridine difference spectra were recorded with $Na_2S_2O_4$-reduced microsomal suspensions (2.7 ml) and 0.3 ml of either 0.5*M* aqueous pyridine or water added to the cuvets.

The wavelength of cytochrome P-450 (or P-448) maximum absorption was measured on a Cary 14 spectrophotometer calibrated with a holmium filter.

Statistics

Results are quoted as the mean ± S. E. M. and were analyzed by Student's *t*-test except where the nature of the results necessitated the use of a ranking test.

Results and Discussion

Effect of Dioxin Administration on the *In Vivo* Action, and *In Vitro* Metabolism of Drugs in the Rat

One or 3 days after being given a single oral dose of dioxin rats show a shortening of the zoxazolamine paralysis time but an increase in the duration of action of hexobarbitone (*2*). The effect on the hexobarbitone sleeping time becomes progressively more marked with time until, 2 weeks after dosing, rats sleep over 4 hr and some of them die without waking (Table 1).

Table 1. Effect of dioxin on the hexobarbitone sleeping time of male rats.[a]

Time after dosing	Sleeping time, min [b]		Increase over controls, *P*	
	Dioxin (200 μg/kg, PO)	Solvent	%	
12 hr	32.9 ± 3.5 (6)	37.7 ± 3.3 (5)	—	NS
24 hr	40.0 ± 3.1 (6)	27.4 ± 1.8 (6)	46	<0.01
72 hr	75.5 ± 5.9 (6)	33.6 ± 3.3 (6)	125	<0.001
1 week	125.6 ± 14.1 (6)	21.5 ± 2.9 (5)	480	<0.001
2 weeks	> 244 (6)	25.2 ± 2.7 (6)	870	0.0022 [c]

[a] Hexobarbitone sodium administered as described in Materials and Methods section.
[b] Numbers of animals in parentheses.
[c] By ranking test.

In view of the prolonged reduction of food intake of rats dosed with dioxin (*3*) it is possible that a starvation effect (*13*) might contribute significantly to the increased hexobarbitone sleeping times at later stages in the intoxication (1 or 2 weeks after dosing). However earlier experiments (*2*) had suggested that, 1 day after dosing with dioxin, the reduced food intake was not the sole cause of the prolongation of the hexobarbitone sleeping time. This interpretation is open to criticism, since in these experiments the animals had not been prefasted before dioxin administration and the dioxin might have slowed down the absorption of the residual food in the stomach and intes-

tine. Accordingly groups of eight female rats were starved for 39 hr and then dosed with dioxin (200 μg/kg, PO) or oil. Following 24 hr further starvation the hexobarbitone sleeping time was measured; the value for the dosed group, 89.0 ±3.8 min, was significantly higher than that of the control group, 63.2 ±5.3 min, ($P < 0.005$). Thus the early effect of dioxin on the sleeping time cannnot be entirely due to differences in food consumption or absorption between control and treated animals. Therefore in all following experiments we have used rats which had been given a single dose of dioxin (200 μg/kg) 1 or 3 days previously.

It was considered possible that dioxin might prolong the hexobarbitone sleeping time by altering either the sensitivity of the nervous system or the distribution of the barbiturate within the body. These possibilities were ruled out by an experiment in which the sleeping times of groups of dioxin-treated and control rats were measured and, immediately after their waking, blood was collected for the analysis of plasma barbiturate levels (*8*). Table 2 indicates that, although there was a significant increase in the sleeping time of the dosed animals, the waking plasma barbiturate levels of the two groups were not significantly different.

Table 2. Effect of dioxin on the sleeping time and waking plasma barbiturate level of female rats.[a]

Treatment	*N*	Sleeping time, min	Plasma barbiturate, μg/ml
Dioxin	6	99.3 ± 19.8	67.9 ± 2.2
Controls	6	45.9 ± 4.2	64.5 ± 2.9
P		<0.025	NS

[a] Rats were dosed with dioxin (200 μg/kg, PO) or oil and 3 days later the sleeping time induced by hexobarbitone sodium (75 mg/kg, IP) was measured. Immediately after waking the animals were anesthetized (ether) and blood collected from the heart. Plasma barbiturate was measured by the method of Chromy and Babjuk (*8*).

Three days after a single oral dose of either dioxin or oil to male or female rats the liver microsomes were isolated and incubated with an NADPH-generating system and either hexobarbitone or zoxazolamine (Table 3). With either sex, following dioxin treatment, there was a significant increase in the amount of zoxazolamine metabolized, and with males there was a significant decrease in the quantity of hexobarbitone oxidized. With the microsomes from female rats the control level of hexobarbitone metabolism was lower than the males and, although in the treated livers it was lower still, the difference was not significant. Apart from this, the results agree with the *in vivo* experiments and indicate that dioxin modifies the duration of the pharmacological action of both zoxazolamine and hexobarbitone by changing the rate of metabolism of these drugs by the liver microsomes.

Table 3. Effect of dioxin treatment on the metabolism of hexobarbitone and zoxazolamine by rat liver microsomes.[a]

Treatment	Sex	Zoxazolamine metabolized, nmole/mg microsomal protein/hr[b]	Hexobarbitone metabolized, nmoles/mg microsomal protein/hr[b]
Dioxin	M	32.2 ± 4.2 (10)	31.0 ± 23.9 (5)
Controls	M	15.7 ± 2.0 (10)	192.0 ± 18.7 (5)
		P <0.005	<0.001
Dioxin	F	46.9 ± 7.9 (5)	29.4 ± 47.8 (5)
Controls	F	6.3 ± 5.4 (5)	109.2 ± 49.7 (5)
		P <0.005	NS

[a] Rats (180–200 g) received dioxin (200 μg/kg) or oil PO and were killed 3 days later. Microsomes were isolated by the calcium/sucrose method (see Materials and Methods) and the assays performed essentially as described elsewhere (*9–12*).

[b] Numbers of animals in parentheses.

Effect of Dioxin Administration on the Liver Content and Spectral Properties of Microsomal Cytochrome P-450

Besides oxygen and NADPH, it is known that three other components are required for the reconstitution of a system capable of metabolizing drugs *in vitro*. These are a lipid, phosphatidyl choline; a flavoprotein, NADPH/cytochrome P-450 reductase, and a hemoprotein, cytochrome P-450 (or P-448) (*14–16*). We have investigated the effect of dioxin treatment on cytochrome P-450 of rat liver microsomes. Groups of male

Table 4. Changes in liver weight and microsomal protein and cytochrome P-450 induced by dioxin treatment of male rats.[a]

Treatment	Weight wet liver, g/100 g BW	Microsomal protein, mg/g liver	Microsomal cytochrome P-450		
			nmoles/g liver	nmoles/mg protein	λ_{max} (nm)
Dioxin	4.34 ± 0.12	26.5 ± 0.9	55.1 ± 1.5	2.08 ± 0.07	447.6 ± 0.04
Controls	3.21 ± 0.06	27.3 ± 0.6	28.1 ± 0.7	1.03 ± 0.02	449.6 ± 0.08
	P <0.001	NS	<0.001	<0.001	<0.001

[a] Rats received dioxin (200 μg/kg, PO) or oil and 3 days later were killed and liver microsomes isolated at 105,000g (see Materials and Methods). Each value is the mean of five observations.

rats received an oral dose of either dioxin or oil and three days later were killed and their liver microsomes isolated. Table 4 indicates that there was a significant increase in the weight of wet liver from the treated animals. There was no increase in the microsomal protein content, expressed per gram of tissue, but the cytochrome content doubled. Further, the wavelength of the peak maximum of the cytochrome spectrum was shifted from 450 nm to 448 nm. Such a shift is a feature of the microsomal enzyme induction brought about by compounds such as 20-methylcholanthrene (*17*). Since this inducer stimulates the metabolism of aromatic compounds, including zoxazolamine (*18*) this change correlates well with the observed stimulation of zoxazolamine metabolism both *in vivo* and *in vitro*. However it should be remembered that, if the CO/cytochrome P-448 complex has an extinction coefficient more than four times that of P-450, as has been reported (19), then there has in fact been a decrease in hemo-protein concentration.

The interaction of the reduced cytochrome P-450 of liver microsomes with the ligands ethyl isocyanide or pyridine is known to result in the formation of a difference spectrum with two peaks in the region 400–460 nm (*20*). The relative intensity of these two peaks, related to the absorption at 500 nm, is dependent on the pH of the suspending medium (20). In the case of liver microsomes prepared from animals pretreated with 20-methylcholanthrene and therefore containing P-448 rather than P-450, the curve of pH dependence of the peak height ratio is so shifted that the peaks are of equal intensity at a lower pH (*21*). The pH dependence of the pyridine difference spectra of microsomes from methylcholanthrene-, dioxin- or oil-treated rats was measured and is shown in Figure 1. It can be seen that the curves due to dioxin or methylcholanthrene treatment are similar to each other but distinct from that of the control.

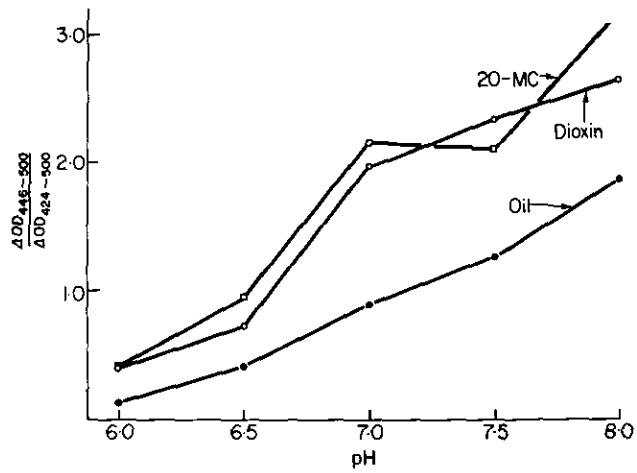

FIGURE 1. Rats (male, 180–200 g) received dioxin (200 μg/kg, PO) or Arachis oil (2 ml/kg, PO) 3 days before killing. 20-Methylcholanthrene was injected (20 mg/kg, 10 mg/ml in oil, IP) 3 and 2 days before killing. All were starved for 24 hr before decapitation. Portions of liver microsomes equivalent to 0.4 g of wet liver were isolated by the calcium/sucrose method (5) and suspended in 0.1M PO_4 buffer (10 ml, containing 1mM Na_2EDTA) of the appropriate pH. Pyridine difference spectra were measured as stated in Materials and Methods.

It is known that, in the oxidized state, the cytochrome P-450 of liver microsomes will interact with various substrates of the drug metabolizing system to produce characteristic difference spectra (*22*). We have studied the effect of dioxin pretreatment of rats on the interaction of aniline and hexobarbitone with rat liver microsomes. With aniline the control microsomes showed a normal Type II spectral change; this was intensified in the case of the microsomes from treated rats (Fig. 2). Such a change is consistent with the increased cytochrome P-448 in these preparations. However, a double reciprocal plot of the spectral change/nmole of P-450 (P-448) against aniline concentration indicated that there was an increase in K_s (binding affinity constant) and in the maximal Type II spectral change (Fig. 3). A similar effect has been reported following methylcholanthrene induction (*23*).

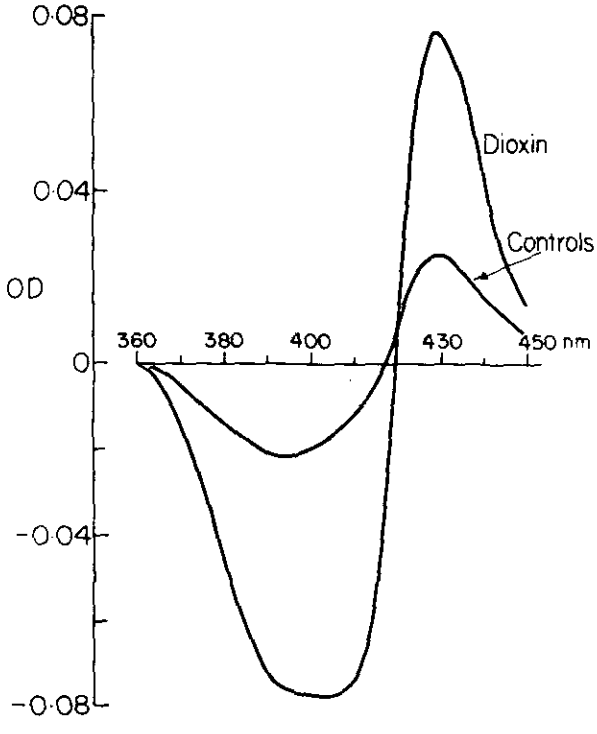

FIGURE 2. Rats (male, 180–200 g) received dioxin (200 μg/kg) or oil PO 3 days before killing. They were starved for 24 hr before isolation of liver microsomes at 105,000g. Preparations from two animals were combined. Difference spectra (aniline 0.41*mM* final concentration) measured as described in Materials and Methods on suspensions containing microsomes equivalent to 0.073g wet liver/ml.

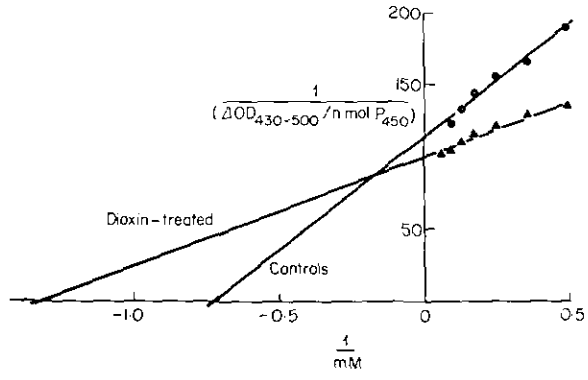

FIGURE 3. Rats (male, 170–190 g) received dioxin (200 μg/kg) or oil PO 3 days before killing. Liver microsomes, equivalent to 2.8 g wet liver, were isolated by the calcium/sucrose method (*5*) and were suspended in 0.1*M* PO_4 buffer (25 ml, pH 7.4) containing 1*mM* EDTA. Aniline concentrations in the cuvet (see Materials and Methods) were varied from 2.0 to 15.2*mM*. Each point is the mean of observations on five animals. On each axis the mean intercepts for groups of dosed and control animals were significantly different ($P < 0.05$). An extinction coefficient of 91/*mM*-cm (*7*) was used in the estimation of cytochrome P-450 (-448). The spectral changes observed are related to the total cytochrome content of the cuvet.

The interaction of hexobarbitone with microsomes from control animals produced the expected Type I difference spectra (*22*) as illustrated in Figure 4. However the microsomal preparations from rats which had received dioxin 3 days previously consistently gave a difference spectrum with a peak at 412 nm and a trough at 380 nm. This type of spectrum has been termed a modified Type II spectral change (*22*) and has been observed following methylcholanthrene pretreatment (*24*), but only with microsomal preparations from female rats. A decrease in the intensity of the Type I difference spectrum due to hexobarbitone has been reported for methylcholanthrene-treated male and female rats (*23*).

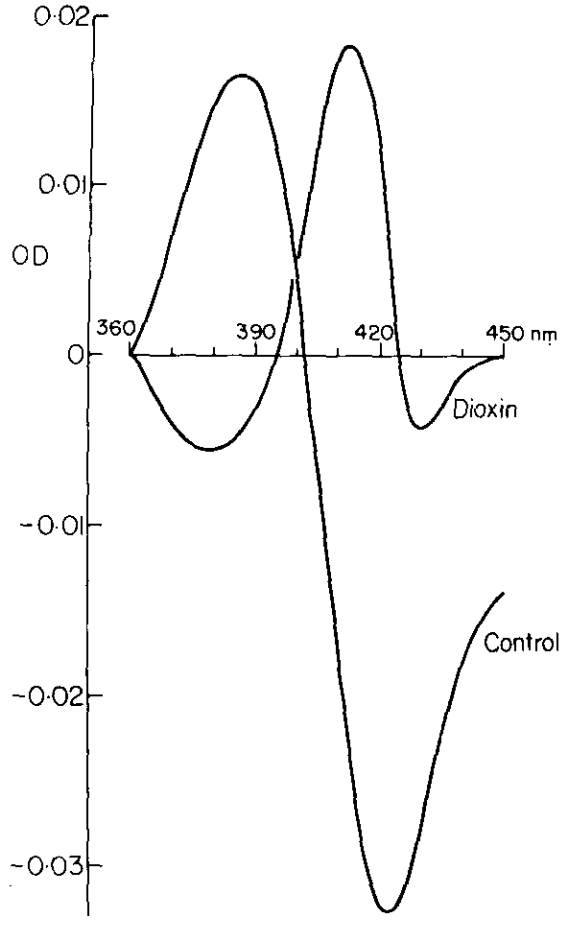

FIGURE 4. All details as in the legend to Figure 2 except that hexobarbitone was added to the sample cuvetts to a concentration of 0.79*mM*.

Comparison of the Effects of 20-Methylcholanthrene and Dioxin on Zoxazolamine Action in Mice of the DBA/2 Strain

All the changes of the properties of the liver microsomes from dioxin-treated rats are consistent with dioxin being a powerful inducer of the methylcholanthrene type. It has been reported that in mice of the DBA/2 strain the levels of hepatic aryl hydrocarbon hydroxylase are unaffected by methylcholanthrene (*25*) or benz[*a*]anthracene (*26*) treatment. Indeed when DBA/2 mice (male, 20–30 g) were injected with methylcholanthrene or oil as described by Nebert et al. (*25*) we found, 24 hr after injection, no significant difference between the zoxazolamine paralysis times of the treated and control groups (58.0 ±5.1 min, $N = 6$ and 59.3 ± 3.9 min, $N = 6$, respectively). This contrasts with C57BL/6 mice (male, 18–26 g) in which methylcholanthrene pretreatment significantly reduced the paralysis time (dosed: 15.0 ± 1.0 min, $N = 9$, controls: 79.5 ± 5.3 min, $N = 9$; $P < 0.0001$). However in both strains a single oral dose of dioxin (200 μg/kg) given 3 days before zoxazolamine significantly lowered the paralysis time (DBA/2, male, dosed: 10.5 ± 0.8 min, $N = 6$, controls: 65.7 ± 4.2 min, $N = 5$, $P < 0.001$; for C57BL/6 see Table 5).

Table 5. Effect of dioxin on sleeping and paralysis times of C57 BL/6 mice.[a]

Treatment	Sex	Sleeping time, min[b] After 3 days	After 10 days	After 20 days	Paralysis time after 3 days, min
Dioxin	M	52.6 ± 6.2 (7)	60.6 ± 4.7 (7)	>159.7 (7)	7.0 ± 0.7 (4)
Oil	M	71.3 ± 3.8 (6)	60.2 ± 7.7 (4)	53.4 ± 7.3 (6)	46.8 ± 3.5 (5)
		P <0.05	NS	<0.01[c]	<0.001
Dioxin	F	52.6 ± 6.5 (7)	—	—	5.9 ± 0.6 (5)
Oil	F	65.1 ± 8.4 (7)	—	—	37.7 ± 4.0 (7)
		P NS			<0.001

[a] Mice (11–27 g) received dioxin (200 μg/kg) or oil PO and hexobarbitone sodium or zoxazolamine after the stated intervals.
[b] Numbers of animals in parentheses.
[c] By ranking test.

Assuming that, under these conditions, the duration of zoxazolamine action is entirely governed by the rate of metabolism in the liver it would appear that the apparent genetic noninducibility of hepatic aryl hydrocarbon hydroxylase in certain mouse strains is not an absolute trait but dependent on the inducer used. This is in agreement with the observations (*26*) that this enzyme, although present in smaller amounts in extrahepatic tissues, is inducible in such organs of strains in which the liver enzyme is unaffected.

Investigation of the Relationship between Stimulation of Zoxazolamine Metabolism and Inhibition of Hexobarbitone Metabolism

Although in the rat dioxin produces simultaneous and divergent effects on the metabolism of zoxazolamine and hexobarbitone by liver microsomes, it is not known whether these two effects are related to each other. An alternative would be that dioxin has two separate and distinct effects: the induction of zoxazolamine hydroxylase and the depression of hexobarbitone oxidase. The following two experiments indicate that, after dioxin treatment, a stimulation of zoxazolamine metabolism can be observed in the absence of any inhibition of hexobarbitone metabolism.

In mice of the C57BL/6 strain, as in the rat, a marked reduction of the zoxazolamine paralysis time was observed at 3 days after dioxin treatment. However, at this time the hexobarbitone sleeping time was either unchanged (in female mice) or shortened (in male mice); a prolongation was seen in males only at 20 days (Table 5).

Table 6. Effect of ethionine on dioxin-induced alterations in sleeping time and paralysis time in rats.[a]

Sex	Route of administration			Sleeping time, min[b]	Paralysis time, min[b]
	Dioxin	Oil	Ethionine		
M	IP	—	—	75.4 ± 13.9 (5)[c]	—
	—	IP	—	36.0 ± 2.2 (5)	—
M	IP	—	PO	41.3 ± 3.6 (5)	84.0 ± 6.5 (5)[d,e]
	—	IP	PO	45.0 ± 3.1 (5)	>383 (5)
M	PO	—	IP	44.7 ± 3.1 (6)	—
	—	PO	IP	42.0 ± 3.4 (6)	—
F	IP	—	PO	83.0 ± 9.4 (6)[f]	119.2 ± 9.4 (6)[e,f]
	—	IP	PO	136.4 ± 7.2 (6)	>480 (6)

[a] Animals received *dl*-ethione (200, 100, and 100 mg/kg) at $t = 0$, 4, and 8 hr, respectively; dioxin (200 μg/kg) or oil at $t = 0.5$ hr and sleeping or paralysis times were measured at $t = 24.5$ hr. All were starved during the experiment.
[b] Numbers of animals in parentheses.
[c] Significantly different from controls at $P < 0.05$.
[d] Significantly different from controls at $P < 0.01$.
[e] By ranking test.
[f] Significantly different from controls at $P < 0.005$.

The effect of dioxin on hexobarbitone metabolism can also be suppressed in rats by administering *dl*-ethionine together with the dioxin. Along with a single oral dose of dioxin male rats were given a series of injections of *dl*-ethionine, an inhibitor of protein synthesis which has been reported as preventing the induction of drug metabolising enzymes by either 20-methylcholanthrene (*27*) or phenobarbitone (*28*). It was found (Table 6) that, whereas the ethionine was ineffective in preventing the reduction of paralysis time in dioxin-treated animals, it did abolish the prolongation of hexobarbitone sleeping time due to dioxin treatment. When the routes of administration

of dioxin and ethionine were interchanged there was still no difference in hexobarbitone metabolism.

When the experiment was carried out with female rats, in which ethionine is a more effective inhibitor of protein synthesis (*29*), the duration of both paralysis and sleep of the dioxin-treated animals was shorter than that of the controls (Table 6). Thus although these doses of ethionine are not effective in blocking the induced metabolism of zoxazolamine (*27*) in either sex, they are capable of preventing or even reversing the effect of dioxin on hexobarbitone metabolism.

Conclusions

1. The divergent effects of dioxin on the duration of action of hexobarbitone and zoxazolamine in the rat *in vivo* (*2*) are a consequence of changes in hepatic metabolism of these drugs.

2. Dioxin causes alterations in the properties of cytochrome P-450 of rat liver microsomes which are similar to those produced by methylcholanthrene.

3. It is the most effective stimulator of aromatic hydroxylation known, see (*1*), and can apparently overcome a genetic resistance to hepatic microsomal enzyme induction in mice of the DBA/2 strain.

4. The effects of dioxin on hexobarbitone and zoxazolamine metabolism can be separated by the use of C57BL/6 mice or ethionine-treated rats and might be due to two separate modes of action.

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