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INTERACTIONS OF SPIRONOLACTONE WITH HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME SYSTEMS*

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Abstract—The nature of the stimulation of hepatic microsomal drug-metabolizing enzymes by spironolactone was studied in male mice. Mice given spironolactone (100 mg/kg, i.p.) twice daily for 3 consecutive days exhibited increased liver weight and microsomal protein content. The administration of spironolactone also stimulated the microsomal metabolism of hexobarbital *in vitro*, the disappearance of hexobarbital from the whole body of mice *in vivo* and reduced hexobarbital sleeping time. Increases were noted in the microsomal *N*-demethylation of ethylmorphine, oxidation of hexobarbital and hydroxylation of aniline. Moreover, prior treatment with spironolactone increased the levels of several components of the microsomal electron transport system which included NADPH-oxidase, NADPH-cytochrome *c* reductase, cytochrome P-450 and NADPH-cytochrome P-450 reductase. In addition, the stimulatory effects of ethylmorphine or hexobarbital on the rate of cytochrome P-450 reduction by NADPH were greater in microsomes from spironolactone-treated animals.

In other studies, it was found that the addition of spironolactone to liver microsomes (1) inhibited the *N*-demethylation of ethylmorphine and (2) blocked the stimulatory effect of ethylmorphine on the reduction of cytochrome P-450.

The results indicate that spironolactone is an inducer of hepatic microsomal drug-metabolizing enzymes in male mice. A proposed mechanism for spironolactone's interaction with these enzyme systems is discussed.

SELYE and co-workers[1,2] have reported that pretreatment with spironolactone, a steroidal diuretic, antagonizes a variety of toxicological and/or pharmacological actions of many drugs. Other studies[3,4] have shown that drug metabolism is increased in animals given spironolactone for several days. In a preliminary communication,[5] we observed an increase in the liver microsomal metabolism of hexobarbital and ethylmorphine after pretreatment with this drug. It was suggested that the ability of spironolactone to reduce the responses of other drugs may be related to an enhancement of their metabolism.

It is well known that a variety of structurally diverse drugs are able to induce microsomal enzyme systems.[6,7] These inducers have been divided into three general classes (a) phenobarbital type; (b) polycyclic hydrocarbons, such as 3-methylcholanthrene, and (c) steroids. The inductive effects of steroids are generally associated with compounds possessing one or more of the following properties: androgenic, progestational, anabolic or glucocorticoidal actions. In this regard, the steroid spironolactone is unique because it is virtually devoid of these endocrine actions.[8]

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In this paper, experiments were designed to examine (a) the nature of liver enzyme induction by spironolactone and (b) the interaction of spironolactone with liver-microsomal drug metabolizing enzyme systems, *in vitro*.

MATERIALS AND METHODS

Animals and pretreatment. Male albino Swiss mice, weighing 20–30 g were used. All animals were maintained on Purina chow diet and received water *ad lib.* Unless stated otherwise, spironolactone (100 mg/kg) or corn oil vehicle was injected intraperitoneally, twice daily for 3 consecutive days. In all experiments injections were given in a volume of 0·10 ml/10 g body weight. Experiments were initiated 24 hr after the last dose.

Sleeping-time. After the injection of hexobarbital sodium (120 mg/kg, i.p.), the duration of sleeping time was equivalent to the time elapsed between loss of the righting reflex until the animal righted itself three times in 30 sec.

Hexobarbital metabolism. At 15, 30 or 60 min after the injection of hexobarbital sodium (80 mg/kg, i.p.), spironolactone-treated or control animals were stunned and homogenized in 4 vol. of distilled water. The homogenate was filtered through cotton gauze and a 7-ml aliquot of the filtrate was assayed by the method of Cooper and Brodie.[9]

Microsomal enzyme assays. Drug metabolism *in vitro* was determined in an incubation mixture consisting of 50 mM tris-HCl buffer (pH 7·4), a NADPH-generating system (5 mM $MgCl_2$, 0·33 mM NADP, 5 mM glucose-6-phosphate, 2 enzyme units glucose-6-phosphate dehydrogenase), 5 mg microsomal protein and substrate, in a final volume of 3·0 ml. After the addition of ethylmorphine (5 mM), hexobarbital (0·67 mM) or aniline (6·67 mM), the reaction mixtures were placed in a Dubnoff metabolic incubator and incubated in air at 37° with shaking (90 cycles/min) for the times indicated in the tables. The method of Nash[10] was used to estimate the amount of formaldehyde formed from ethylmorphine. The conversion of aniline to *p*-aminophenol was assayed by the procedure described by Kato and Gillette[11] and the metabolism of hexobarbital was determined by the procedure of Cooper and Brodie.[9]

Cytochrome P-450 content. The assay for cytochrome P-450 was estimated as described by Omura and Sato.[12] The amount of cytochome P-450 was determined from the absorbance difference (A_{450}–A_{490}) and the molar extinction coefficient of 91 mM^{-1} cm^{-1}.

NADPH-cytochrome c reductase. The reductase activity was measured by the method of Phillips and Langdon[13] using a molar extinction coefficient of 19·1 mM^{-1} cm^{-1}.

NADPH-cytochrome P-450 reductase. The procedure employed was essentially that described by Davies *et al.*[14] and Gigon *et al.*[15] The reduction of cytochrome P-450 by NADPH was assayed at room temperature as follows: carbon monoxide (deoxygenated by passage through an alkaline dithionite solution) was bubbled for 5 min through 2·5 ml of a microsomal suspension (4 mg protein/ml in 0·02 M tris–HCl buffer, pH 7·4, containing 1·15 per cent KCl) in an anaerobic spectrophotometric cell. A plunger assembly containing 50 μl of a NADPH-generating system was fitted to the cuvette and carbon monoxide was then flushed through the inlet for an additional 3 min. After the cuvette was sealed and placed in the sample chamber of a Gilford 240 spectrophotometer, the plunger was depressed and the change in absor-

bance of the CO–cytochrome P-450 complex recorded. Reduction rates were calculated from the initial linear phase of the curve to determine the effect of various substrates on NADPH–cytochrome P-450 reductase. Spironolactone was dissolved in 95 per cent ethanol and added in a volume of 50 μl. Substrates and/or equivalent volumes of ethanol were added to the microsomal suspension during the initial gassing procedure.

Protein determination. Microsomal protein was estimated by the method of Lowry *et al.*[16] with crystalline bovine serum albumin as a standard.

NADPH-oxidase. After the addition of NADPH (0·33 mM) to a 3·0 ml reaction mixture containing 5 mg microsomal protein and 60 μmoles of tris–HCl buffer (pH 7·4), the oxidation of NADPH was determined by measuring the decrease in absorbancy at 340 mμ.

Statistics. Statistical comparisons were made using the Student *t*-test.

RESULTS

Effect of spironolactone pretreatment on hexobarbital sleeping time and the in vivo *and* in vitro *metabolism of hexobarbital.* As shown in Table 1, the administration of spironolactone to male mice reduced the hexobarbital sleeping time to about one-sixth that of the control group. In addition, it was observed that the liver microsomal metabolism of hexobarbital was increased 109 per cent in mice given spironolactone. An enhancement of barbiturate metabolism after spironolactone pretreatment was also observed in the *in vivo* study shown in Fig. 1. At the times indicated spironolactone-treated mice metabolized hexobarbital 1·4–2·1 times more rapidly than the control animals.

TABLE 1. EFFECT OF SPIRONOLACTONE PRETREATMENT IN MALE MICE ON HEXOBARBITAL SLEEPING TIME AND METABOLISM OF HEXOBARBITAL BY LIVER MICROSOMES

Treatment*	Sleeping time† (min ± S.D.)	Hexobarbital metabolism† (m-μmoles/mg prot./20 min)
Control	83 ± 19	29·8 ± 6·3
Spironolactone	15 ± 7	62·3 ± 9·1
% of control	18‡	209‡

* Mice were injected with spironolactone (100 mg/kg, i.p. twice daily) or corn oil for 3 consecutive days.

† All animals received hexobarbital sodium, 120 mg/kg, i.p. (n = 10 for each treatment group). The metabolism of hexobarbital in liver microsomes was carried out as described in Materials and Methods. Values represent the mean ± S.D. of five determinations.

‡ $P < 0·001$.

These preliminary observations suggested that the reduction in sleeping time after pretreatment with spironolactone was mediated by a stimulation of hexobarbital metabolism in liver microsomes.

Influence of prior treatment with spironolactone on liver weight, microsomal protein and various microsomal enzyme levels. In Table 2, it was found that the liver weight, liver–body weight ratio and microsomal protein content were significantly elevated

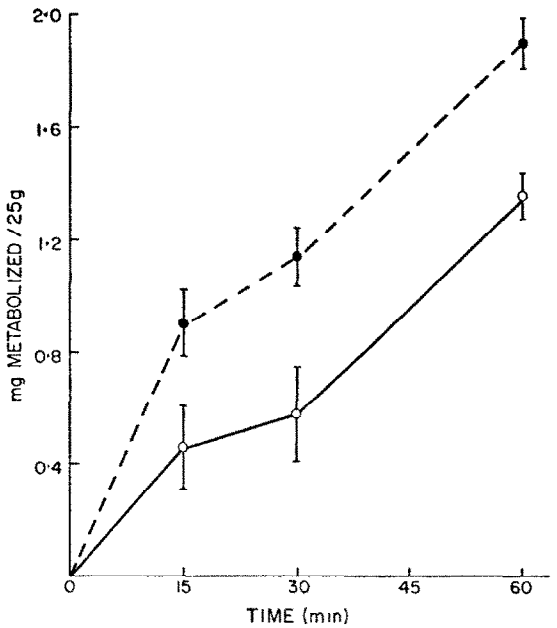

FIG. 1. Influence of spironolactone pretreatment on the *in vivo* metabolism of hexobarbital in male mice. The values plotted represent the mean (mg hexobarbital metabolized/25 g body wt.) ± S.D. of five animals/group at the times indicated after the injection of hexobarbital sodium (80 mg/kg, i.p.). Key: spironolactone-treated (● - - - ●) and control animals (○———○).

in the drug-treated mice as compared to control animals. Moreover, the possible diuretic effect of spironolactone did not appear to be a major contributory factor. In studies involving several hundred mice, the differences in body weight between control and drug-treated animals never exceeded 5 per cent. In addition to the elevation in hexobarbital metabolism, prior treatment with spironolactone increased the microsomal *N*-demethylation of ethylmorphine (154 per cent) and hydroxylation of aniline (30 per cent) (Table 2). Spironolactone pretreatment also increased many components of the electron transport system in liver microsomes. The microsomal reduction of cytochrome *c* and cytochrome P-450 by NADPH increased 75 and 84 per cent respectively, while the cytochrome P-450 content increased 57 per cent. Moreover, the oxidation of NADPH was elevated 23 per cent in liver microsomes isolated from drug-pretreated animals.

Effect of substrates on the reduction of cytochrome P-450. Since treatment with spironolactone significantly increased the reduction of cytochrome P-450 by NADPH, it was of interest to examine the actions of hexobarbital, ethylmorphine and aniline on this enzyme system. As shown in Table 3, ethylmorphine and hexobarbital, both of which produce Type I spectral changes, significantly increased the rate of cytochrome P-450 reduction by NADPH in liver microsomes from control and drug-treated mice. In experiments with aniline, a compound which produces a Type II spectral change, a decrease in the rate of cytochrome P-450 reduction by NADPH was observed in both microsomal preparations. It should also be noted that prior treatment with spironolactone increased the relative rates of cytochrome P-450

TABLE 2. CHANGES IN LIVER WEIGHT, MICROSOMAL PROTEIN CONTENT AND VARIOUS MICROSOMAL ENZYMES AFTER THE TREATMENT OF MALE MICE WITH SPIRONOLACTONE*

Treatment†	Liver weight (g)	Liver/body weight (%)	Microsomal protein content (mg/g liver)	Aniline hydroxylase (m-μmoles *p*-amino-phenol formed/mg prot./15 min)	Ethylmorphine *N*-demethylase (m-μmoles HCHO formed/mg prot./12 min)	NADPH-oxidase (m-μmoles/mg prot./min)	Cytochrome *c* reductase (m-μmoles reduced/mg prot./min)	Cytochrome P-450 (m-μmoles mg prot.)	NADPH-cytochrome P-450 reductase (m-μmoles/mg prot./min)
Control	1·36 ± 0·12	5·39 ± 0·24	24·16 ± 1·10	28·9 ± 2·3	123 ± 9	10·0 ± 0·6	97 ± 4	0·75 ± 0·02	1·91 ± 0·22
Spironolactone	1·61 ± 0·09	6·47 ± 0·27	28·29 ± 1·07	37·4 ± 0·6	312 ± 4	12·3 ± 0·6	170 ± 7	1·18 ± 0·02	3·49 ± 0·40
% of Control	118§	120§	117§	130§	254§	123‡	175§	157§	184§

* Values are expressed as the mean ± S.D. of five determinations. Each individual determination was obtained by pooling livers from four to five animals.
† Animals received spironolactone (100 mg/kg) or corn oil, i.p., twice a day for 3 consecutive days.
‡ $P < 0.01$.
§ $P < 0.001$.

TABLE 3. EFFECT OF HEXOBARBITAL, ETHYLMORPHINE AND ANILINE ON THE REDUCTION OF CYTOCHROME P-450 BY NADPH IN MOUSE LIVER MICROSOMES

Compound	NADPH-cytochrome P-450 reductase* (m-μmoles/mg prot./min)		
	Control	Spironolactone	% of Control
None	2·00 ± 0·28	3·48 ± 0·35	174†
Hexobarbital (5 mM)	5·05 ± 0·56	10·26 ± 0·84	203†
Ethylmorphine (5 mM)	3·92 ± 0·77	9·26 ± 1·34	236†
Aniline (1 mM)	1·25 ± 0·22	2·76 ± 0·53	221†

* Animals received spironolactone (100 mg/kg) or corn oil, i.p. twice daily for 3 consecutive days. Experiments were carried in liver microsomes as described in Materials and Methods. Results represent the mean of four to five determinations ± S.D.
† $P < 0·05$.

reduction to the same degree in the presence of aniline, ethylmorphine or hexobarbital (103–136 per cent). Therefore, it is evident that the substrate-mediated increases in these reduction rates do not adequately explain the observed differences in their microsomal metabolism (Tables 1 and 2). However, the substrate-mediated increases in the rates of cytochrome P-450 reduction with ethylmorphine or hexobarbital (Table 4) paralleled the 2·1–2·5-fold elevation in their microsomal metabolism after pretreatment with spironolactone. The differences in the reduction rates of the cytochrome by the Type I substrates (hexobarbital and ethylmorphine) were 2·2–3·0-fold greater in liver microsomes from drug pretreated mice. In contrast, the net decrease in the rate of cytochrome P-450 reduction by aniline, a Type II substrate, was not appreciably altered by spironolactone treatment.

Inhibition of microsomal N*-demethylation by spironolactone*, in vitro. Several inducers have been shown to inhibit drug metabolism when added *in vitro*. Thus, it was of interest to determine the influence of spironolactone on the microsomal

TABLE 4. DIFFERENCES IN THE REDUCTION RATES OF CYTOCHROME P-450 BETWEEN CONTROL AND SPIRONOLACTONE-INDUCED MICROSOMES IN THE PRESENCE OF TYPE I AND TYPE II SUBSTRATES

Pretreatment	Net change in the rates of cytochrome P-450 reduction* (m-μmoles/mg prot./min)		
	Type I		Type II
	Hexobarbital	Ethylmorphine	Aniline
Control	3·05	1·92	−0·75
Spironolactone	6·78	5·78	−0·72
Ratio (spironolactone/control)	2·23	3·01	0·96

* Absolute values for these calculations appear in Table 3. The tabulated values were obtained by determining the difference in the reductase activity before and after the addition of each substrate in control or induced microsomes (i.e. Aniline: $\Delta_{control} = 1·25 - 2·00 = -0·75$).

TABLE 5. EFFECT OF SPIRONOLACTONE ON THE *N*-DEMETHYLATION OF ETHYLMORPHINE IN SPIRONOLACTONE-INDUCED AND CONTROL MICROSOMES FROM MALE MICE

Treatment*	Ethylmorphine *N*-demethylation† (m-μmoles HCHO formed/mg prot./12 min)				
	Control	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Corn oil	144·0 ± 5·8 (0)	136·0 ± 5·0 (6)	105·3 ± 7·0 (27)	54·7 ± 5·5 (62)	31·0 ± 1·0 (79)
Spironolactone	322·7 ± 15·0 (0)	314·4 ± 10·1 (3)	267·3 ± 8·3 (17)	130·0 ± 6·0 (60)	63·3 ± 2·3 (80)

* Animals received spironolactone (100 mg/kg) or corn oil, i.p., twice daily for 3 consecutive days.
† Values are expressed as the mean ± S.D. of triplicate determinations and the results in parenthesis represent the percentage inhibition of ethylmorphine *N*-demethylation in the presence of varying concentrations of spironolactone. Spironolactone incubations were carried out as described in Materials and Methods.

N-demethylation of ethylmorphine. As shown in Table 5, the addition of spironolactone in concentrations of 10^{-3}–10^{-5} M effectively antagonized the metabolism of ethylmorphine. Moreover, there was no apparent difference in the relative inhibition by spironolactone in liver microsomes from control or drug-pretreated animals.

Effect of spironolactone on NADPH-cytochrome P-450 reductase. The influence of spironolactone on the reduction of cytochrome P-450 by NADPH is given in Table 6. With the highest concentration of spironolactone (0·5 mM), only slight inhibition of the reductase activity was noted. However, this compound was able to block the increase in the reduction of the cytochrome by ethylmorphine. In the presence of

TABLE 6. EFFECT OF SPIRONOLACTONE ON NADPH-CYTOCHROME P-450 REDUCTASE IN MOUSE LIVER MICROSOMES

Compounds tested*	Initial rate of cytochrome P-450 reduction by NADPH† (m-μmoles reduced/mg prot./min)	% of control
Experiment I		
1. Control	1·76 ± 0·23	100
2. SL, 0·5 mM	1·62 ± 0·16	92
3. EtM, 5·0 mM	3·57 ± 0·38‡	203
4. EtM, 5·0 mM + SL, 0·5 mM	2·60 ± 0·17‡§	148
Experiment II		
1. Control	1·81 ± 0·22	100
2. SL, 0·5 mM	1·65 ± 0·33	91
3. An, 0·5 mM	1·24 ± 0·22‡	69
4. An, 0·5 mM + SL, 0·5 mM	1·03 ± 0·14‡	57
5. An, 1·0 mM	0·90 ± 0·23‡	50
6. An, 1·0 mM + SL, 0·5 mM	0·84 ± 0·22‡	46

* Abbreviations: spironolactone (SL), ethylmorphine (EtM) and aniline (An).
† Values represent the mean ± S.D. of four to five determinations in the microsomal preparation. The rates of cytochrome P-450 reduction by NADPH in the presence or absence of the compounds were carried out as described in Materials and Methods.
‡ Values significantly different ($P < 0·05$) from corresponding control values.
§ Values were significantly different ($P < 0·05$) in the samples 3 vs. 4, Experiment I.

spironolactone, the ethylmorphine-mediated increase in reductase activity was decreased by 54 per cent (compare 3 vs. 4 in Experiment I). On the other hand, the decrease in the rate of cytochrome P-450 reduction mediated by aniline was unaffected by the presence of spironolactone (compare 3 vs. 4 and 5 vs. 6 in Experiment II). These observations demonstrate that spironolactone is able to selectively modify the effect of ethylmorphine on cytochrome P-450 reduction.

DISCUSSION

In recent years, studies have greatly clarified the mechanism of drug oxidation by NADPH-dependent liver-microsomal enzymes.[17] As indicated in Fig. 2, it has been proposed that the electron donor, NADPH, reduces cytochrome *c* reductase which directly or indirectly reduces the hemoprotein, ferricytochrome P-450 to ferrocytochrome P-450. This terminal oxidase, in turn, complexes with drug and oxygen to form the oxidized drug and ferricytochrome P-450. At present, considerable emphasis

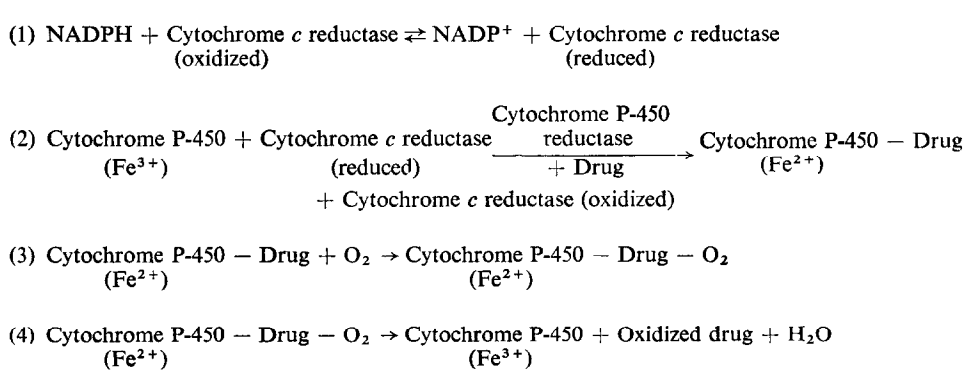

FIG. 2. A proposed mechanism of drug oxidations in liver microsomes.

has been placed on studies designed to elucidate whether the ferricytochrome P-450 combines with the substrate before reduction or if it is reduced prior to combination with the drug. It is well-known that increased drug-metabolizing enzyme activity in liver microsomes generally correlates with an elevation in one or more components of the electron transport system, namely NADPH-cytochrome *c* reductase, cytochrome P-450, and NADPH-cytochrome P-450 reductase.[6,7,14,15,18]

In the present study, we have observed significantly elevations in NADPH-oxidase, cytochrome P-450 and reductase activities for cytochrome P-450 and cytochrome *c* after pretreatment with spironolactone in mice. These results, coupled with the increased microsomal metabolism of ethylmorphine, hexobarbital and aniline after spironolactone treatment are similar to the induction of liver enzyme systems mediated by barbiturates, e.g. phenobarbital.[17]

A comparison of these findings with those of Stripp *et al.*[4] indicates that there is a marked sex and species difference in the nature of spironolactone induction between rats and mice. In rats, they observed increases in the activity of NADPH-cytochrome *c* reductase and ethylmorphine *N*-demethylase; no significant changes were reported

in cytochrome P-450 or in the reduction of cytochrome P-450 by NADPH. Moreover, they found that the barbiturate-sleeping times were reduced only in female rats. Recent findings[19] in our laboratory suggest that the prior administration of spironolactone to mice greatly reduced the hexobarbital sleeping time (6-fold) in both sexes. Similarly, increases were noted in cytochrome P-450, cytochrome *c* reductase, ethylmorphine *N*-demethylase and hexobarbital metabolism in liver microsomes.

A qualitative, but not quantitative agreement was observed between hexobarbital metabolism *in vivo* and *in vitro* and sleeping time, e.g. 2- and 6-fold changes respectively. In view of these results, it might be suggested that spironolactone could modify the redistribution of the barbiturate. In this regard, the diuretic action of spironolactone did not appear to play a major role in the termination of hexobarbital sleeping time in mice. The body weights of animals given spironolactone were never less than 95 per cent that of the control animals.

Several investigators have shown that the addition of various compounds to a suspension of liver microsomes produces two types of changes in the absorption spectrum; these substrates are referred to as Type I (e.g. ethylmorphine, hexobarbital) and Type II (e.g. aniline).[20,21] Recently, Davies *et al.*[14] and Gigon *et al.*[15] have presented evidence indicating that the substrate-mediated increase in the rate of cytochrome P-450 reduction by NADPH is related to the maximal rate of ethylmorphine *N*-demethylation. In their kinetic studies, Gigon *et al.* studied the influence of Type I and Type II substrates on the reduction of cytochrome P-450 by NADPH in liver microsomes from male rats; Type I substrates increased the reductase activity whereas Type II compounds decreased the reduction rate of the cytochrome. In this paper, the observed stimulation of NADPH–cytochrome P-450 reductase by ethylmorphine and hexobarbital and inhibition by aniline serves to substantiate these earlier findings.[15,18]

In our studies, treatment with spironolactone increased the rate of cytochrome P-450 reduction to the same degree by Type I and II substrates. Moreover, the changes in the reduction of the cytochrome by Type I substrates were similar to the changes in the overall activity of the drug metabolizing enzymes. In addition, it can be seen that the elevation in the metabolism of ethylmorphine and hexobarbital is closely related to the substrate-mediated increase in the rates of cytochrome reduction between control and drug-induced microsomal preparations (Table 4). The lack of a similar correlation with aniline agrees favorably with the concept that the rate-limiting step in the metabolism of Type I, but not Type II substrates, is the reduction of the cytochrome P-450–substrate complex.[18,22]

Other lines of evidence indicated that the interaction of spironolactone with liver microsomes was more selective for the Type I substrates: (1) the microsomal metabolism of ethylmorphine and hexobarbital was found to be 3–5 times greater than that observed with aniline (Tables 1 and 2) and (2) the addition of spironolactone to a microsomal suspension blocked the effect of ethylmorphine, but not aniline, on the reduction of cytochrome P-450 by NADPH (Table 6). In the latter studies, the inability of spironolactone to modify the action of aniline suggests that spironolactone may be acting at a terminal site in the electron transport system, e.g. at a site subsequent to NADPH-cytochrome *c* reductase (see Fig. 2). According to the current view of the electron flow in liver microsomes, spironolactone would be expected to inhibit both Type I and Type II substrates if acting at a site prior to cytochrome P-450. In

this regard, the metabolism of aniline and/or the effect of this substrate on the reduction of cytochrome P-450 may be mediated through a different sequence of events or by a different cytochrome P-450 in liver microsomes.

The evidence presented in this paper demonstrates that spironolactone pretreatment enhances the activity of microsomal drug-metabolizing enzyme systems, and that this effect may play a major role in the attenuation of pharmacological and toxicological responses of other drugs. If spironolactone possesses little or no anabolic activity, as has been suggested previously, it is a unique inducer of drug metabolism.

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