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Role of the Steroid 17α-Hydroxylase in Spironolactone-Mediated Destruction of Adrenal Cytochrome P-450

DAVID C. KOSSOR, SHIRO KOMINAMI, SHIGEKI TAKEMORI, and HOWARD D. COLBY

Department of Pharmacology and Toxicology, Philadelphia College of Pharmacy and Science, Philadelphia, Pennsylvania 19104 (D.C.K., H.D.C.), and Faculty of Integrated Arts and Sciences, Hiroshima University, Higashisenda-Machi, Naka-Ku, Hiroshima 730, Japan (S.K., S.T.)

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SUMMARY

Previous investigations have established that spironolactone (SL) is converted to a reactive metabolite by adrenal microsomal enzymes, resulting in the degradation of cytochrome P-450 (P-450). Deacetylation of SL to 7α-thiospironolactone (7α-thio-SL) is the first step in the activation pathway, but further NADPH-dependent metabolism of 7α-thio-SL is required for P-450 destruction. Studies were done to evaluate the role of the steroid 17α-hydroxylase in the activation of 7α-thio-SL by adrenal microsomes. Incubation of guinea pig adrenal microsomes with 7α-thio-SL in the presence of NADPH effected >50% declines in P-450 content and in 17α-hydroxylase activity but no change in the rate of 21-hydroxylation. Preincubation of the microsomes with antisera to the 17α-hydroxylase P-450 isozyme (P-450$_{17\alpha,lyase}$) decreased 17α-hydroxylase but not 21-hydroxylase activity and prevented the degradation of P-450 by 7α-thio-SL. Control IgG had no effect on 17α-hydroxylase activity or on the 7α-thio-SL-mediated destruction of P-450. When added to a purified P-450$_{17\alpha,lyase}$ preparation, 7α-thio-SL and the endogenous substrate progesterone caused typical type I spectral changes, but SL did not. Incubation of a purified and reconstituted 17α-hydroxylase system, consisting of P-450$_{17\alpha,lyase}$, NADPH-P-450 reductase, cytochrome b_5, and dilauroylphosphatidylcholine, with 7α-thio-SL plus NADPH effected the complete degradation of the P-450$_{17\alpha,lyase}$. Neither progesterone nor SL caused P-450 destruction with the reconstituted enzyme preparation. The results provide direct evidence for the activation of 7α-thio-SL by the 17α-hydroxylase and support the hypothesis that a mechanism-based inhibition of the enzyme occurs. The data also provide additional evidence that 7α-thio-SL is an obligatory intermediate in the degradation of P-450 by SL.

SL, a widely used diuretic, has been shown to inhibit steroidogenesis in the adrenal cortex and testes (1–11). The mechanism of action in both organs appears to involve degradation of P-450, causing declines in steroid hydroxylase activities (2–6). The results of *in vitro* studies indicate that the actions of SL on P-450 are mediated by a reactive microsomal metabolite of the drug (5, 6, 12). Deacetylation of SL to 7α-thio-SL is an obligatory first step in the activation pathway in adrenal microsomes (Fig. 1), but further metabolism of 7α-thio-SL is required for P-450 degradation (12).

Indirect evidence tends to implicate the steroid 17α-hydroxylase in the conversion of 7α-thio-SL to the ultimate reactive species responsible for enzyme degradation. P-450 destruction by 7α-thio-SL is highly correlated with 17α-hydroxylase activity under a variety of experimental conditions (13, 14). In addition, there is a rapid and relatively selective decline in 17α-hydroxylase activity after 7α-thio-SL metabolism by adrenal or testicular microsomes *in vitro* (5, 6, 13). Taken together, these observations suggest that 7α-thio-SL may be a suicide inhibitor of the 17α-hydroxylase. The studies presented in this communication were done to pursue this hypothesis by directly evaluating the role of the 17α-hydroxylase in the degradation of P-450 by 7α-thio-SL.

Materials and Methods

Adult male English Short Hair guinea pigs (1000–1200 g) were obtained from Camm Research Institute (Wayne, NJ) and maintained under standardized conditions of light (6:00 a.m. to 6:00 p.m.) and temperature (22°), on a diet of Purina laboratory chow and water *ad libitum*. All animals were allowed at least 7 days to become acclimated to the housing conditions before use in experiments. Animals were sacrificed by CO_2 inhalation, and adrenal glands were quickly removed and placed in cold 1.15% KCl, 0.05 M Tris·HCl (pH 7.4), on ice. Adrenals were bisected longitudinally, and the dark-brown inner zone, consisting primarily of zona reticularis, was gently dissected from the tan outer zone, which is composed of the zona fasciculata and zona glomerulosa (15, 16). Only outer zone tissue was used for the experiments described in this report, because of the relative abundance of 17α-hydroxylase activity in that zone (17) and because of the greater destruction of P-450 by SL in outer versus inner zone microsomal preparations (13). All steps in the preparation of subcellular fractions were carried out at 0–4°. Tissues were homogenized with a motor-driven glass-Teflon homogenizer in KCl-Tris buffer. The homogenates

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ABBREVIATIONS: SL, spironolactone; 7α-thio-SL, 7α-thiospironolactone; P-450, cytochrome(s) P-450.

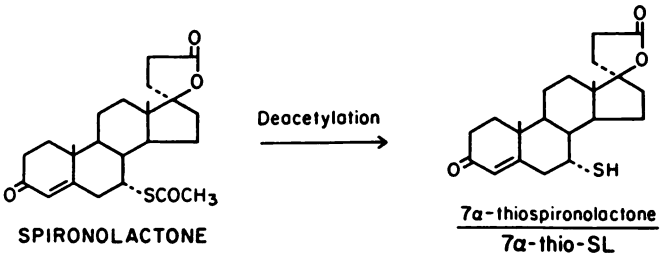

Fig. 1. Chemical structures of SL and its deacetylated metabolite 7α-thio-SL.

were centrifuged first at 900 × *g* for 10 min, followed by 9000 × *g* for 20 min. The 9000 × *g* pellet was washed once and recentrifuged at 9000 × *g* for 20 min. The supernatants of the 9000 × *g* centrifugations were further centrifuged at 105,000 × *g* for 75 min, in a Beckman L8-60M ultracentrifuge, to obtain the microsomal pellets.

Incubation conditions for studies on the degradation of microsomal P-450 by 7α-thio-SL were essentially the same as described previously (12), except that the temperature was maintained at 4°. The lower temperature was used in order to decrease the rate of P-450 degradation, thereby allowing for better control of experimental conditions. Microsomal suspensions (0.5 mg of protein/ml) in 1.15% KCl, 0.05 M Tris·HCl, containing 3 mM $MgCl_2$, were incubated with 7α-thio-SL (0.1 mM) and/or an NADPH-generating system (0.4 mM $NADP^+$, 10 mM sodium isocitrate, 0.3 units/ml isocitrate dehydrogenase) under air, with shaking, for the times indicated. For each of the incubation conditions employed, one set of flasks served as unincubated or time 0 controls. The initiation of incubations (by addition of 7α-thio-SL or vehicle) was staggered to permit immediate processing of samples for P-450 and/or steroid hydroxylase assays after appropriate incubation times.

The anti-$P\text{-}450_{17\alpha,lyase}$ IgG was prepared from rabbit serum and elicited with purified $P\text{-}450_{17\alpha,lyase}$ from guinea pig adrenal microsomes, as described previously (18). Preparation included ammonium sulfate precipitation, DE-52 column chromatography, and gel filtration with G-200. Samples were dissolved in 0.9% NaCl containing 20 mM Tris·HCl (pH 8.0). Control IgG was similarly prepared from preimmune serum. For inhibition of microsomal 17α-hydroxylase activity, aliquots of microsomal suspensions were preincubated with anti-$P\text{-}450_{17\alpha,lyase}$ (or control IgG), at a ratio of 5 mg of IgG/mg of microsomal protein, for 2 hr at 4°, as described previously (19). The preincubations had no effects on P-450 concentrations, and preincubations in the absence of anti-$P\text{-}450_{17\alpha,lyase}$ did not affect steroid hydroxylase activities. After the preincubations, aliquots of the microsomal suspensions were taken for incubations with 7α-thio-SL and/or NADPH, as described above.

The reconstituted 17α-hydroxylase/lyase enzyme system employed was identical to that previously described (20, 21). For the reconstitution, 10–20 pmol of $P\text{-}450_{17\alpha,lyase}$ were mixed with equivalent amounts of NADPH-P-450 reductase and of cytochrome b_5 in 30 mM potassium phosphate buffer (pH 7.2), containing 20% glycerol, 0.1 mM EDTA, and 75 μM dilauroylphosphatidylcholine, and then preincubated at 0° for 30 min. The $P\text{-}450_{17\alpha,lyase}$ was purified from guinea pig adrenal microsomes, in the presence of 10 μM progesterone for stabilization, as described previously (20). NADPH-P-450 reductase was prepared from bovine adrenal microsomes, and cytochrome b_5 from guinea pig adrenal microsomes (20, 21). The reconstituted enzyme preparation was diluted with appropriate amounts of incubation medium immediately before use in experiments.

Steroid 17α-hydroxylase activity was assayed as the rate of conversion of progesterone to 17α-hydroxyprogesterone plus 11-deoxycortisol, and 21-hydroxylase activity was measured as the conversion of progesterone to 11-deoxycorticosterone plus 11-deoxycortisol. After incubation of adrenal microsomes or the reconstituted 17α-hydroxylase with steroids and/or NADPH, aliquots of the incubation mixtures were diluted 1:2 with enzyme assay buffer (17). Enzyme assays were done essentially as described previously (17) but were scaled down to accommodate smaller amounts of protein. Microsomal assays were done with 0.025 mg of protein, in a total volume of 0.625 ml, and were incubated for 45 min. Assays with the reconstituted enzyme preparation contained 5 pmol of $P\text{-}450_{17\alpha,lyase}$, in a total volume of 0.25 ml, and were incubated for 30 min at 37°. In all cases, incubation conditions were used that ensured linearity of product formation with respect to incubation time and protein concentrations. After incubation, steroids were extracted with ethyl acetate, and metabolites were separated by reverse phase high performance liquid chromatography, as described previously (17); metabolites were quantified by UV absorbance at 254 nm.

P-450 was measured as the dithionite-reduced CO complex, by the method of Omura and Sato (22). Steroid-induced type I difference spectra were recorded with an Aminco DW-2a spectrophotometer in the split-beam mode. Microsomal protein concentrations were determined by the method of Lowry *et al.* (23), with bovine serum albumin as the standard. Data are presented as means ± standard errors. Statistical analyses of differences between group means were done with Student's *t* test or the Newman-Keuls multiple range test, as appropriate.

Results and Discussion

Incubation of guinea pig adrenal microsomes with 7α-thio-SL plus an NADPH-generating system at 4° for 60 min effected approximately a 50% loss of P-450 (Table 1). The same incubation conditions caused a significant decline in steroid 17α-hydroxylase activity but had no effect on the rate of 21-hydroxylation (Table 1). In the absence of 7α-thio-SL or of NADPH, there were no changes in P-450 content or in 17α-hydroxylase activity. These effects are similar to those previously obtained when incubations were done at 37° (5, 6, 12), but degradation proceeded at a much slower rate at the lower temperature. As shown in Fig. 2, at 4° the degradation of P-450 by 7α-thio-SL continued for at least 60 min. In contrast, at 37° the loss of P-450 in the same microsomal preparations reached a maximum (60–70% loss) within 5–10 min (data not shown). At 37°, like at 4°, 7α-thio-SL caused inactivation of the 17α-hydroxylase but not of the 21-hydroxylase.

The observations described above serve to explain why we have previously had difficulty in obtaining reproducible time-course data for 7α-thio-SL-mediated P-450 degradation. In prior studies, incubations were routinely done at 37° and "terminated" by placing of flasks in an ice bath (4°). However, the latter procedure apparently is not sufficient to prevent the further degradation of P-450, and the overall loss of P-450 depends partly upon how long samples are kept on ice before analyses are initiated. Thus, for accurate time-course data, samples must be assayed immediately after incubation or gassed

TABLE 1

Effects of incubation of adrenal microsomes with 7α-thio-SL and/or an NADPH-generating system on P-450 content and on 17α-hydroxylase and 21-hydroxylase activities

Values are means ± standard errors of four experiments and are expressed as percentage of unincubated values.

Incubation conditions	P-450[a]	17α-Hydroxylase[b]	21-Hydroxylase[c]
	%	%	%
Control	103 ± 6	97 ± 4	89 ± 4
NADPH	95 ± 9	103 ± 2	95 ± 4
7α-Thio-SL	105 ± 3	104 ± 12	92 ± 9
NADPH + 7α-thio-SL	57 ± 3[d]	47 ± 5[d]	92 ± 13

[a] 100% equivalent to 1.3 nmol/mg of protein.
[b] 100% equivalent to 12.0 nmol/min × mg of protein.
[c] 100% equivalent to 3.9 nmol/min × mg of protein.
[d] $p < 0.05$ (versus corresponding control value).

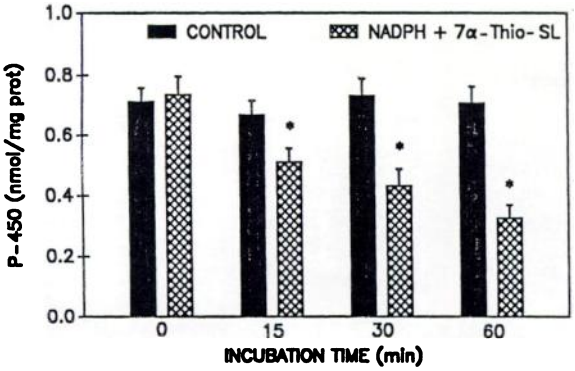

Fig. 2. Time-course for the loss of P-450 resulting from the incubation of adrenal microsomes at 4° with 7α-thio-SL plus an NADPH-generating system. Incubations were done and P-450 concentrations were determined as described in Materials and Methods. Controls are samples incubated with neither 7α-thio-SL nor the NADPH-generating system. Values are means ± standard errors of six experiments. *, $p < 0.05$ versus corresponding control value.

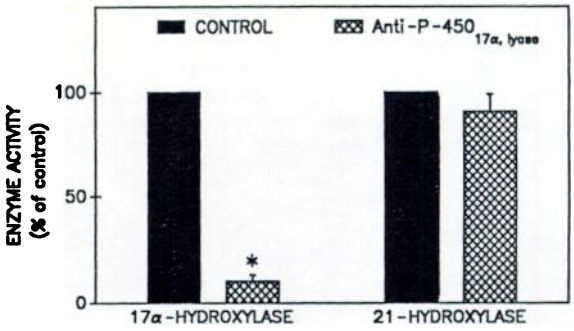

Fig. 3. Effects of anti-P-$450_{17\alpha,lyase}$ IgG on adrenal microsomal 17α-hydroxylase and 21-hydroxylase activities. Microsomal preparations were preincubated with or without anti-P-$450_{17\alpha,lyase}$ IgG, and enzyme activities were then determined as described in Materials and Methods. Values are means ± standard errors of four experiments and are expressed as percentage of the corresponding control value; 100% was equivalent to 9.5 ± 1.3 (17α-hydroxylase) or 2.9 ± 0.1 (21-hydroxylase) nmol/min × mg of protein. *, $p < 0.05$ versus corresponding control value.

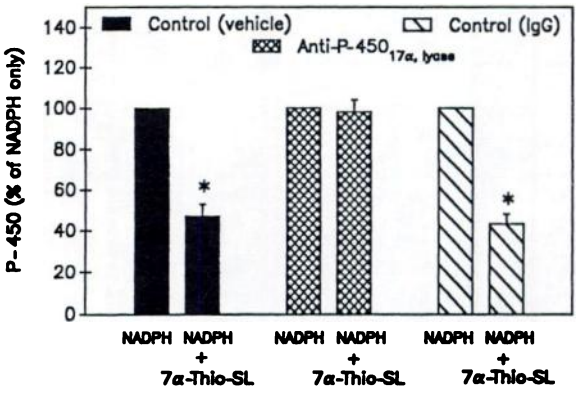

Fig. 4. Effects of anti-P-$450_{17\alpha,lyase}$ and control IgG on the 7α-thio-SL-mediated degradation of P-450 in adrenal microsomes. Microsomal suspensions were preincubated with or without anti-P-$450_{17\alpha,lyase}$ IgG or control IgG and then incubated with an NADPH-generating system in the presence or absence of 7α-thio-SL, as described in Materials and Methods. Values are means ± standard errors of four experiments and are expressed as percentage of the corresponding NADPH-only values; 100% was equivalent to 1.1, 0.9, and 1.2 nmol/mg of protein for vehicle control, anti-P-$450_{17\alpha,lyase}$, and control IgG experiments, respectively. *, $p < 0.05$ versus corresponding NADPH-only value.

with CO to prevent further enzymatic activation of 7α-thio-SL. The latter cannot be done if steroid hydroxylase activities are to be determined, because the CO inhibits P-450-mediated steroid metabolism. Addition of progesterone to the samples after incubation prevents further activation of 7α-thio-SL, by acting as a competitive substrate (13), and allows for subsequent measurement of 17α- and 21-hydroxylase activities with progesterone as the substrate. However, the interactions of various substrates with P-450 cannot then be evaluated because of the large amounts of progesterone present. We have used each of the approaches described above, at various times, to evaluate the metabolism-dependent effects of 7α-thio-SL on P-450 degradation, and the results obtained were always similar to those shown in Fig. 2 and Table 1. For the studies described in this report, the method used was to initiate assays immediately after incubation. In addition, all microsomal incubations with 7α-thio-SL were done at 4°, because the slower degradation of P-450 at the lower temperature allowed for more accurate assessment of antibody effects on the rate of degradation.

To directly assess the role of the steroid 17α-hydroxylase in the microsomal activation of 7α-thio-SL, the effects of anti-P-$450_{17\alpha,lyase}$ IgG on 7α-thio-SL-mediated P-450 degradation were evaluated. As shown in Fig. 3, preincubation of adrenal microsomes with anti-P-$450_{17\alpha,lyase}$ caused a >90% decline in 17α-hydroxylase activity but had no significant effect on 21-hydroxylation (19), the other major microsomal P-450-catalyzed reaction. As a result of preincubation with the anti-P-$450_{17\alpha,lyase}$, the subsequent degradation of microsomal P-450 by 7α-thio-SL was completely prevented (Fig. 4). In the same microsomal preparations preincubated without anti-P-$450_{17\alpha,lyase}$ or with control IgG (Fig. 4), 7α-thio-SL effected >50% degradation of P-450. Accompanying the destruction of P-450 were significant declines in 17α-hydroxylase activities (data not shown). These observations strongly suggest that the P-$450_{17\alpha,lyase}$ is involved in the activation of 7α-thio-SL, resulting in P-450 degradation. To further pursue this hypothesis, experiments were done with a purified and reconstituted 17α-hydroxylase/lyase preparation.

The reconstituted enzyme system catalyzed the conversion of progesterone to 17α-hydroxyprogesterone and Δ^4-androstenedione only, the expected products of the P-$450_{17\alpha,lyase}$ (data not shown). The 21-hydroxylated products typically resulting from microsomal progesterone metabolism were not produced by the reconstituted preparation, demonstrating its specificity. As previously reported (20), incubation of the reconstituted enzyme preparation in the absence of steroid substrate (NADPH alone) caused some P-450 degradation (Table 2). Thus, the smaller decline in P-450 content after incubation with 7α-thio-SL alone than with NADPH alone (Table 2) is indicative of protection by the steroid. However, when the reconstituted 17α-hydroxylase/lyase was incubated with 7α-thio-SL in the presence of NADPH, total degradation of the P-$450_{17\alpha,lyase}$ occurred (Table 2). In contrast, neither SL nor progesterone, in the presence of NADPH, caused the degradation of P-$450_{17\alpha,lyase}$ (Table 2). 17α-Hydroxylase activities were proportionately similar to P-450 levels after incubation of the reconstituted enzyme and NADPH with progesterone, SL, or 7α-thio-SL (Table 3). Thus, 7α-thio-SL caused complete loss of enzyme activity, but substantial activity remained after incubation with progesterone or SL. These data demonstrate that 7α-thio-SL is activated by the reconstituted 17α-hydrox-

TABLE 2

Effects of incubation of a reconstituted 17α-hydroxylase/lyase preparation with 7α-thio-SL, SL, or progesterone and/or an NADPH-generating system on P-450$_{17\alpha,lyase}$ concentrations

Values are means ± standard errors of triplicate determinations with the reconstituted enzyme preparation and are expressed as percentage of unincubated (time 0) control values; steroid concentrations were 50 μM.

Preincubation conditions	P-450$_{17\alpha,lyase}$[a]
	% of control
NADPH	62 ± 6
7α-Thio-SL	81 ± 5
NADPH + 7α-thio-SL	<1[b]
NADPH + SL	64 ± 5
NADPH + progesterone	82 ± 5

[a] 100% equivalent to 5.0 pmol.
[b] Below detectable levels in all experiments.

TABLE 3

Effects of incubation of a reconstituted 17α-hydroxylase/lyase preparation with progesterone, SL, or 7α-thio-SL plus an NADPH-generating system on enzyme activity

Values are means ± standard errors of quadruplicate determinations with the reconstituted enzyme preparation; steroid concentrations were 50 μM.

Preincubation conditions	17α-Hydroxylase activity
	nmol/min × nmol of P-450
NADPH + progesterone	16.3 ± 0.6
NADPH + SL	13.9 ± 0.1
NADPH + 7α-thio-SL	<1.0[a]

[a] Activity was below detectable levels in all experiments.

ylase/lyase, resulting in the destruction of P-450. They also indicate that SL does not directly cause enzyme inactivation, providing further evidence that deacetylation to 7α-thio-SL is a prerequisite for the degradation of P-450.

Additional evidence for the conclusions described above was obtained from studies on substrate binding to the purified P-450$_{17\alpha,lyase}$. As shown in Fig. 5, addition of the endogenous substrate progesterone to the P-450$_{17\alpha,lyase}$ produced the expected type I spectral change indicative of substrate binding to P-450. Similar difference spectra were induced by 7α-thio-SL (Fig. 5), but only a very small spectral change was demonstrable with SL and it did not appear to be concentration dependent (Fig. 5). The spectral data suggest that 7α-thio-SL is a good substrate for the P-450$_{17\alpha,lyase}$ but that SL is at best a poor substrate and they are consistent with the hypothesis that SL must be deacetylated before metabolism by the 17α-hydroxylase/lyase occurs (12).

The results provide direct evidence for the activation of 7α-thio-SL by the 17α-hydroxylase/lyase and the consequent degradation of the enzyme. In conjunction with earlier observations (5, 6, 12, 13), these findings indicate that a mechanism-based (suicidal) inhibition of the enzyme is involved. Previous investigations (5, 6, 12–14) have demonstrated that enzyme inactivation by 7α-thio-SL follows pseudo-first-order kinetics, is saturable, and requires substrate metabolism. In addition, the 17α-hydroxylase is protected by natural substrates for the enzyme but not by nucleophiles such as reduced glutathione. Thus, many of the criteria required for establishing suicide inhibition of the 17α-hydroxylase by 7α-thio-SL have now been satisfied.

Most prior studies, as well as the data presented here, have focused on the actions of 7α-thio-SL on adrenal microsomal P-450 isozymes. It is likely that the same mechanism is applicable to effects on testicular P-450, because the P-450$_{17\alpha,lyase}$ is the

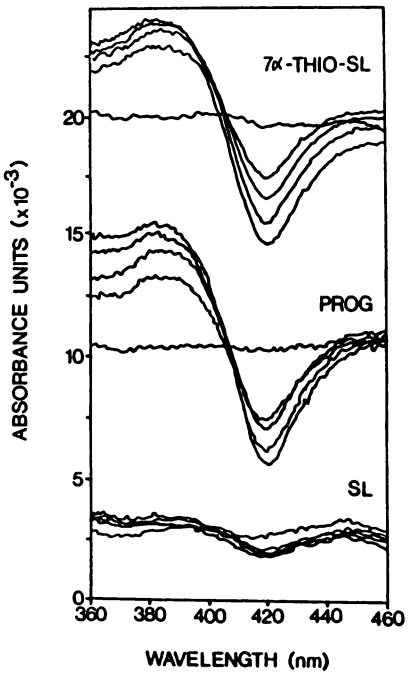

Fig. 5. Interactions of progesterone (*PROG*), SL, and 7α-thio-SL with purified P-450$_{17\alpha,lyase}$. Suspensions of the P-450$_{17\alpha,lyase}$ at a concentration of 85 nM were divided equally between two cuvettes in an Amino DW-2a recording spectrophotometer. After a baseline of equal light absorbance was recorded, steroids were added to the sample cuvette in small volumes of ethanol, and equal amounts of ethanol only were added to the reference cuvette. Steroid concentrations in the sample cuvette after each successive addition were 10, 25, 50, and 100 μM; the final addition caused a maximal spectral change for each steroid. Spectra were recorded at room temperature.

major isozyme in testes microsomes. However, it has been demonstrated by Decker *et al.* (24, 25) that SL and 7α-thio-SL also cause the metabolism-dependent degradation of hepatic P-450 in dexamethasone-pretreated rats (24, 25). Because the P-450$_{17\alpha,lyase}$ is not among the isozymes found in hepatic microsomes, different enzymes and/or mechanisms must be involved in the degradation of hepatic and adrenal P-450. In liver, like adrenal, deacetylation of SL to 7α-thio-SL appears to be an obligatory first step in the activation pathway (25). Decker *et al.* (24, 25) have presented evidence suggesting that 7α-thio-SL is a suicide inhibitor of P-450$_p$ in rat hepatic microsomes, but further investigation is needed to unequivocally determine the validity of their hypothesis.

Although the *in vitro* actions of SL (or 7α-thio-SL) on adrenal microsomes are specific for 17α-hydroxylase/lyase inactivation, administration of the drug to animals *in vivo* decreases the activities of other microsomal and mitochondrial monooxygenases as well (1–4). Additional studies are needed to determine the mechanism(s) responsible for these additional actions of SL *in vivo*. It is possible that such changes occur secondarily to the degradation of P-450$_{17\alpha,lyase}$, with attempts at resynthesis ultimately causing overall adrenal heme depletion, or are caused by extraadrenal metabolites of the drug. Pursuit of these as well as other possibilities should contribute to a fuller understanding of the mechanisms responsible for some of the side effects of SL on steroidogenesis.

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Send reprint requests to: Howard D. Colby, Department of Pharmacology and Toxicology, Philadelphia College of Pharmacy and Science, 600 South 43rd Street, Philadelphia, PA 19104.