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# Secobarbital-Mediated Inactivation of Rat Liver Cytochrome P-450$_b$: A Mechanistic Reappraisal

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**SUMMARY**

Administration of the allylbarbiturate secobarbital (SB) to phenobarbital-pretreated rats is known to result in structural and functional loss of hepatic cytochrome P-450 and generation of *N*-alkylated prosthetic heme derivatives. Isozyme-selective functional markers have led us to confirm P-450$_b$ as the isozyme selectively inactivated by the drug. In contrast to its inactivation by allylisopropylacetamide, such SB-inactivated P-450$_b$ is not amenable to structural and functional repair by exogenous heme, for reasons that remain unclear. In an effort to gain some insight, we have explored various possible mechanisms. In the course of these studies with rat liver microsomes enriched in P-450$_b$ as well as isolated purified P-450$_b$, we have found that, along with prosthetic heme alkylation, a significant fraction of the hemoprotein also undergoes drug-mediated alkylation of the apocytochrome, presumably at the active site. Accordingly, an equimolar ratio of irreversibly bound drug to functionally inactive residual P-450$_b$ chromophore is observed after incubation of the purified isozyme with SB and NADPH. Thus, P-450$_b$-mediated oxidative metabolism of SB appears to partition not only between prosthetic heme alkylation and epoxidation but apoprotein alkylation as well.

The porphyrinogenic potential of various structurally related allyl-containing carbamides such as AIA, allylisopropylacetylcarbamide (Sedormid), and *N*-allylisopropylacetyl *N*-methylcarbamide and of allyl-containing ureides such as SB (Seconal), diallylbarbituric acid (Dial, allobarbital), or allyl-(1-isopropyl)barbituric acid (Aprobarbital) has long been recognized (1–5). It is relatively more recently that the mechanistic basis for the superior porphyrinogenic potential of such compounds over that of their corresponding saturated analogs has been understood. Such inherent superiority apparently resides in the allyl functionality, which may be activated in the course of oxidation by cytochrome P-450[1] to a cation radical (4–8). Subsequent attack by this radical on an accessible pyrrolic N of the cytochrome P-450 heme moiety results in an alkylated porphyrin, the so-called green pigment, thereby destroying the heme moiety and "suicidally" inactivating the cytochrome (8). Because most of the heme synthesized in the liver is committed to cytochrome P-450 formation, and because this commitment is further increased on induction of the cytochrome by various inducers including barbiturates, such mechanism-based destruction of hepatic cytochrome P-450 heme results in dramatic depletion of hepatic heme. Whereas normal individuals compensate for this depletion by turning on *de novo* heme synthesis, individuals with genetically determined lesions in the heme synthetic pathway are compromised and respond with an acute attack of hepatic porphyria.

Suicidal inactivation of rat hepatic cytochrome P-450 by AIA and SB is known to be dramatically enhanced after induction of the hemoprotein by PB (3, 9). Such *in vivo* or *in vitro* destruction of the PB-inducible hepatic microsomal P-450 isozymes may be detected not only spectroscopically but also by the visually discernible green discoloration imparted to the rat liver microsomes and attributable to green pigment formation. Furthermore, we have shown that, after drug-mediated prosthetic heme alkylation, some heme-alkylated isozymes can be structurally and functionally repaired with exogenous heme (10, 11). For instance, after AIA-mediated inactivation, P-450$_b$ (as evidenced by its specific functional marker, PROD is nearly completely restored by *in vivo* heme administration to rats or *in vitro* hemin incubation of liver homogenates (11). Such findings thus reveal that apocytochrome P-450$_b$ is relatively resilient, inasmuch as on regaining fresh heme it can be reassembled to a fully functional holocytochrome and therefore appears to survive AIA-mediated alkylation of its heme.

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[1] The following terms will be used interchangeably throughout the text: "cytochrome P-450" and "P450"; "hemin" and "heme" for iron-protoporphyrin IX, irrespective of its oxidation state.

**ABBREVIATIONS:** AIA, allylisopropylacetamide; PB, phenobarbital; SB, secobarbital [allyl-(1-methylbutyl)-barbituric acid]; PROD, pentoxyresorufin *O*-deethylase; DEX, dexamethasone; APB, aprobarbital; δ-ALA, 100δ-aminolevulinic acid; DLPC, dilauroylphosphatidylcholine; EM, ethylmorphine; BZP, benzphetamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

On the other hand, although SB has been reported to selectively inactivate purified rat liver cytochrome P-450$_b$ (P-450-4) (12) and, *in vivo* as well as *in vitro*, SB-mediated inactivation of PB-inducible hepatic microsomal P-450 isozymes is also associated with "greening" of the liver microsomes, it is unclear whether such inactivation is exclusively due to *N*-alkylation of P-450$_b$ heme. If this were to occur (analogous to the findings with AIA), it was reasonable to expect apocytochrome P-450$_b$ to survive its prosthetic heme alkylation and to be amenable to repair by exogenous heme. We have examined the structural and functional restoration of rat liver microsomal cytochrome P-450$_b$ after its SB-mediated inactivation. Our studies reported here indicate that, unlike its inactivation by AIA, SB-mediated inactivation of this isozyme involves alkylation of its apoprotein in addition to that of its heme moiety, and results in a structurally and functionally irreparable entity.

## Methods

**Animals.** Male Sprague-Dawley rats (200–250 g) obtained from Bantin and Kingman were used throughout these studies. The rats were allowed *ad libitum* intake of food and water. Animals were pretreated with phenobarbital sodium (80 mg/kg, intraperitoneally) daily for 5 days. When SB treatment was indicated, the PB-pretreated rats were given a single injection of the drug (135 mg/kg, subcutaneously, dissolved in glass-distilled water) 24 hr after the last PB injection. The animals were then placed in an $O_2$ chamber, to counteract hypoxia, and sacrificed 1 hr later. Rats were treated with AIA (135 mg/kg, subcutaneously) or APB (135 mg/kg, subcutaneously) exactly as with SB, except that APB was dissolved in NaOH (0.1 N) with mild heating, neutralized, and diluted with glass-distilled water to the appropriate concentration for injection. When DEX pretreatment was indicated, the steroid, dissolved in aqueous Tween-80 (100 mg/kg, intraperitoneally), was injected daily for 4 days.

**Hemin-reconstitution of SB-inactivated cytochrome P-450.** *In vitro* hemin reconstitution was performed exactly as described previously (10) with rat liver homogenates obtained from PB-pretreated rats given SB for 1 hr before killing. We have previously established that such *in vitro* hemin treatment has no effect on any of the basal P-450-dependent activities in PB-pretreated rats (11, 13).

Hepatic cytochrome P-450 content and mixed function oxidase activities were assayed exactly as described previously (11). Microsomal hexobarbital hydroxylase activity was determined radiometrically by the formation of 3′-OH-hexobarbital using a modification of the method of Kupfer and Rosenfeld (14). [2-$^{14}$C]Hexobarbital (5 $\mu$mol, ~50,000 dpm/$\mu$mol) was preincubated in a final volume of 0.8 ml of 0.1 M potassium phosphate buffer, pH 7.4, with NADPH (1 mM), isocitrate (5 mM), isocitrate dehydrogenase (1.5 units), and $MgCl_2$ (5 mM) for 3–5 min at 37°. Microsomes (1 mg of protein) were added and the reaction continued for exactly 8 min, at the end of which it was terminated by addition of ice-cold citrate buffer (1 M) containing 10% (w/v) NaCl, pH 5.5. The metabolites and unreacted hexobarbital were extracted and quantitated as described (14).

**Synthesis of [2-$^{14}$C]SB.** [$^{14}$C]SB was synthesized in three sequential steps as follows.

Step 1. Dimethyl-2-(1-methylbutyl)malonate. Dimethylmalonate (0.022 mol) in methanol was added dropwise to sodium methoxide (0.022 mol), which also was suspended in methanol. 2-Bromopentane (0.022 mol) was then added and the mixture was refluxed for 5 hr. After cooling, sodium methoxide (5 mmol) and 2-bromopentane (0.5 mmol) were added, and the mixture was refluxed for 2 hr. The solution was then diluted with 100 ml of water, acidified with 3 N sulfuric acid, and then extracted with ether. The ether layer was then dried over sodium sulfate and evaporated, and the oily residue was distilled under vacuum to obtain dimethyl 2-(1-methylbutyl)malonate (0.011 mol).

Step 2. [2-$^{14}$C]5-(1-Methylbutyl)barbituric acid. [$^{14}$C]Urea (1 mCi, specific activity 50 mCi/mmol) and unlabeled urea (1.7 mmol) were mixed in ethanol, the solvent was evaporated, and the urea dried first at 60° for 2 hr and then in a vacuum desiccator at room temperature overnight. Sodium methoxide (3.4 mmol) and dimethyl-2-(1-methylbutyl)malonate (1.7 mmol) in methanol were added and the mixture was refluxed for 45 hr. After cooling, the solution was diluted with 60 ml of water, acidified with 3 N sulfuric acid, and then extracted with ether. The organic phase was dried over sodium sulfate and evaporated *in vacuo*. The residue was recrystallized from ethanol to obtain [2-$^{14}$C] 5-(1-methylbutyl)barbituric acid.

Step 3. 5-Allyl-5-(1-methylbutyl)barbituric acid. A trace of copper powder (1–2 mg) was added to [2-$^{14}$C]5-(1-methylbutyl)barbituric acid (0.48 mmol) dissolved in NaOH (1 N, 0.48 ml) and water (1 ml), the mixture was stirred, and allylbromide (0.61 mmol) was added. The mixture was warmed to 35° for 8 min before addition of NaOH (1 N, 0.14 ml) and allylbromide (0.11 mmol). The reaction mixture was kept at 35° for 1 hr with stirring. After cooling, the crystalline material precipitating from the acid solution was extracted with ether. The organic phase was dried over sodium sulfate and the solvent was evaporated *in vacuo*. The product was isolated and purified by thin layer chromatography (silica gel G) with chloroform/acetone (90:10, v/v) as the developing solvent. Its identity as 5-allyl-5-(1-methylbutyl)barbituric acid (0.15 mmol) was confirmed by $^1$H NMR and mass spectrometry.

**[$^{14}$C]SB-alkylation of rat hepatic microsomal protein and purified P-450$_b$.** Covalent binding of [$^{14}$C]SB to microsomal protein was determined after incubating rat liver microsomes (2.4 mg/ml) with [$^{14}$C]SB (~2.5 $\mu$Ci, 0.9 mM), NADPH (1 mM), isocitrate (5 mM), isocitrate dehydrogenase (3 units), EDTA (1.5 mM), and potassium phosphate buffer (0.1 M, pH 7.4) in a final volume of 5 ml. Controls consisted of similar incubations from which NADPH was omitted. In some experiments, bovine serum albumin (20 mg) was included in parallel incubation mixtures. At the end of the incubation, protein was precipitated with trichloroacetic acid (25% w/v) and washed twice with methanol containing 5% $H_2SO_4$ (v/v), followed by two washes of ether/ethanol (1:3, v/v) and one wash with 80% methanol, and processed as described (15).

For determination of [$^{14}$C]SB binding to purified P-450$_b$, hepatic cytochrome P-450$_b$ and NADPH cytochrome P-450 reductase were isolated and purified from PB-pretreated rats exactly as described by Waxman and Walsh (12) and Shephard *et al.* (16), respectively. The incubation mixtures (final volume, 1.6 ml) consisted of P-450$_b$ (≃3.2 nmol), P-450 reductase (3.2 nmol), DLPC (64.3 nmol), [$^{14}$C]SB (~0.6 $\mu$Ci, 1.0 $\mu$mol), EDTA (1.6 $\mu$mol), and NADPH (1.6 $\mu$mol) in chelexed potassium phosphate buffer (0.1 M, pH 7.4), containing 20%, v/v, glycerol, and 20 $\mu$M EDTA. The two enzymes were first preincubated for 10 min in the presence of DLPC before addition of the remaining components and then incubated at 37° for an additional 30 min. Control incubations, which excluded NADPH, were performed in parallel. At the end of 30 min, aliquots (0.6 ml) were taken for determination of P-450 content. To remove noncovalently bound SB, the remainder (with fresh purified P-450$_b$ added as carrier) was applied to a column of Sephadex G-25 (fine) equilibrated with 50 mM Tris-acetate buffer (pH 7.4) containing 20%, v/v, glycerol, 0.5%, w/v, sodium cholate, and 100 $\mu$M EDTA.

Selective binding of [$^{14}$C]SB to P-450$_b$ was confirmed by radioactive quantitation of the incubated proteins after their filtration through Sephadex G-25 and separation by SDS-PAGE and Western blot transfer to nitrocellulose paper. The P-450$_b$ band was identified by immunostaining with rabbit anti-rat P-450$_b$ polyclonal antibodies followed by anti-rabbit horseradish peroxidase-coupled goat IgG and peroxidatic stain development as described previously (11).

To insure complete removal of the SB-alkylated heme from the apocytochrome, SDS (1%, w/v, final) and cholate (0.5% w/v) were added to the incubation mixture, followed by fresh purified P-450$_b$ (~3.0 nmol) as carrier, before passing the incubates through Sephadex G-25 for determination of irreversibly bound [$^{14}$C]SB to the apocyto-

TABLE 1

**Effects of SB treatment and *in vitro* hemin restoration on hepatic cytochrome P-450 content and mixed function oxidase activity in untreated, PB-pretreated, or DEX-pretreated rats**

All values (except where indicated) are mean ± standard deviation obtained from the number of animals indicated in parentheses. For experimental details see Methods.

| Assay | No treatment | | | PB treatment | | | DEX treatment | |
|---|---|---|---|---|---|---|---|---|
| | Control | SB − heme | SB + heme | Control | SB − heme | SB + heme | Control | SB |
| P-450[a] | 1.18 (2) | 1.01 (2) | 1.09 (2) | 2.07 ± 0.39 (24) | 1.25 ± 0.27 (24)[e] | 1.60 ± 0.33 (17)[e,j] | 1.91 ± 0.20 (3) | 1.82 ± 0.12[h] (4) |
| EM[b] | 160 (2) | 142 (2) | 146 (2) | 510 ± 74 (12) | 363 ± 82 (13)[e] | 458 ± 116 (8)[h,j] | 976 ± 74 (3) | 794 ± 55[g] (4) |
| BZP[b] | 75 (2) | 99 (2) | 104 (2) | 433 ± 64 (15) | 263 ± 59 (16)[e] | 367 ± 66 (11)[g,j] | 252 (1) | 242 (2) |
| PCNMA[b] | 83 (2) | 84 (2) | 75 (2) | 106 ± 15 (10) | 84 ± 16 (11)[f] | 93 ± 12 (6)[h,k] | —[i] | — |
| HXB[c] | — | — | — | 78 ± 19 (6) | 50 ± 17 (6)[g] | 64 (2) | — | — |
| PR[d] | — | — | — | 5989 ± 1716 (10) | 1432 ± 369 (9)[e] | 1814 ± 551 (9)[e,k] | — | — |
| ERY[b] | — | — | — | — | — | — | 180 ± 38 (3) | 171 ± 36[h] (4) |

[a] Content, nmol of P-450/mg of protein.
[b] *N*-demethylase, nmol of HCHO formed/mg of protein/15 min. PCNMA, *p*-chloro-*N*-methylaniline.
[c] Hydroxylase, nmol of 3′-OH hexobarbital formed/mg of protein/8 min; HXB, hexobarbital.
[d] *O*-deethylase (PROD), pmol resorufin formed/mg of protein/min.
When statistically analyzed by Students *t* test (two-tailed), values were found to be significantly different from corresponding control values at: [e] $p < 0.001$, [f] $p < 0.01$, [g] $p < 0.02$. [h] Not significantly different.
Significantly different from values from corresponding SB-heme systems at: [j] $p < 0.001$, [j] $p < 0.05$. [k] Not significantly different.
[i] Not determined.

TABLE 2

**Effects of SB, AIA, and APB treatment and hemin restoration on hepatic cytochrome P-450 content and mixed function oxidase activity in PB-pretreated rats**

For experimental details see Methods. All values (except where indicated) are mean ± standard deviation obtained from the number of animals in parentheses.

| Group | Cytochrome P-450 content | Mixed function oxidase activity | | |
|---|---|---|---|---|
| | | EM | BZP | Pentoxyresorufin |
| | *nmol/mg of protein* | *nmol of HCHO/mg of protein/15 min* | | *pmol of resorufin/ mg of protein/min* |
| None | 2.05 ± 0.37 (28) | 494 ± 84 (14) | 420 ± 71 (17) | 6091 ± 1713 (12) |
| SB | | | | |
| No heme | 1.25 ± 0.27 (24)[e] | 363 ± 82 (13)[e] | 263 ± 59 (16)[e] | 1432 ± 369 (9)[e] |
| Heme | 1.60 ± 0.33 (17)[e,e] | 458 ± 116 (8)[d,g] | 367 ± 66 (11)[d,e] | 1814 ± 551 (9)[e,h] |
| AIA | | | | |
| No heme | 0.96 ± 0.09 (3)[e] | —[i] | 140 (1) | 1268 (2) |
| Heme | 1.54 ± 0.15 (3)[c,f] | — | 360 (1) | 6408 (2) |
| APB | | | | |
| No heme | 1.28 ± 0.28 (5)[e] | 293 ± 92 (3)[b] | 241 ± 30 (4)[e] | 2840 (2) |
| Heme | 1.94 (2) | — | 285 (1) | 4212 (2) |

Statistical analyses by Student's *t* test (two-tailed) yielded the following:
Significantly different from corresponding values in PB-treated (none) animals: [e] $p < 0.001$, [b] $p < 0.005$, [c] $p < 0.05$. [d] Not significantly different.
Significantly different from corresponding (no heme) incubated values within each drug-inactivating group:[e] $p < 0.001$; [f] $p < 0.005$; [g] $p < 0.05$. [h] Not significantly different.
[i] Not determined.

TABLE 3

**Estimation of green pigments present in rat liver microsomes after SB-mediated inactivation of P-450**

| Cytochrome P-450 | | | $^3$H-Green Pigments | | |
|---|---|---|---|---|---|
| Specific activity[a] | Loss due to SB[b] | | | | |
| *dpm/nmol* | *nmol/mg of protein* | *total nmol* | *dpm*[c] | *nmol*[d] | *% of P-450 loss* |
| 1860 | 1.14 | 348 | 53,400 | 82 | 24 |

[a] The specific activity was obtained by assuming that all of the radioactivity present in the microsomal preparation was initially associated with cytochrome P-450.
[b] A PB-pretreated rat was given [2,3-$^3$H]δ-ALA. After 2 hr, SB was administered and the animal was sacrificed 1 hr later. A PB control rat given [$^3$H]δ-ALA for 2 hr was used to estimate the initial labeled P-450 content and specific activity.
[c] Total radioactivity of green pigments isolated from 305 mg of microsomal protein.
[d] Value adjusted for 35% recovery.

chrome. The irreversibility of $^{14}$C-binding to P-450$_b$ was established as follows: the incubates were first passed through Sephadex G-25 to remove freely associated [$^{14}$C]SB and the [$^{14}$C]SB-incubated proteins were precipitated with 5% $H_2SO_4$/methanol (v/v) and exhaustively washed with the same solvent mixture. Because such $H_2SO_4$/methanol treatment would not only remove any loosely bound [$^{14}$C]SB that escaped the Sephadex step but also any adventitiously bound [$^{14}$C]SB-alkylated P-450$_b$-heme, these findings indicate that the observed [$^{14}$C]SB binding was exclusively due to an irreversibly linked [$^{14}$C]SB-protein adduct. Furthermore, because the specific $^{14}$C counts after Sephadex G-25 treatment were essentially identical to those obtained after protein precipitation and solvent washes, these results indicate that Sephadex treatment alone is sufficient to remove both loosely associated drug and its derived green pigments.

**SB-mediated P-450-heme alkylation of hepatic microsomal protein.** PB-pretreated rats were given the heme precursor [2,3-$^3$H]δ-ALA (3.3 μCi) or [4-$^{14}$C]δ-ALA (20 μCi) 3 hr before killing. When *in vivo* heme alkylation to hepatic microsomal protein was monitored, rats previously administered [$^3$H]δ-ALA were injected with SB (135 mg/kg, subcutaneously) 1 hr before killing. Hepatic microsomes were prepared and their $^3$H-heme alkylation was monitored exactly as de-

TABLE 4

**Secobarbital-induced degradation of radiolabeled cytochrome P-450 heme to irreversibly bound heme-protein adducts *in vivo* and *in vitro***

Experimental details are given in Methods and in the legend to Table 3.

| *In vivo* | P-450 loss | | | Irreversibly bound $^3$H radioactivity | | | |
|---|---|---|---|---|---|---|---|
| | *nmol/mg of protein* 1.14 | | | *dpm/mg of protein*[a] 203 | *nmol/mg of protein* 0.08 | | *% of SB-mediated P-450 loss* 7 |
| *In vitro incubation system* | $^{14}$*C-Cytochrome P-450*[b] | | | *Irreversibly bound $^{14}$C-radioactivity* | | | |
| | *Initial $^{14}$C-heme*[c] | *P-450 loss*[d] | | *Total*[e] | $^{14}$*C-heme*[f] | | |
| | *nmol* | *%* | *nmol* | *cpm/incubation* | *nmol/incubation* | *%* | *% of SB-mediated $^{14}$C-P-450 loss*[g] |
| Experiment 1 | | | | | | | |
| + NADPH − SB | 12.73 | 6 | 0.76 | 1400 | 0.35 | 2.7 | |
| + NADPH + SB | 12.73 | 28 | 3.56 | 2500 | 0.63 | 4.9 | |
| Loss due to SB | | 22 | 2.80 | | 0.28 | | 10 |
| Experiment 2 | | | | | | | |
| + NADPH − SB | 10.99 | 0 | 0 | 800 | 0.17 | 1.5 | |
| + NADPH + SB | 10.99 | 18 | 2.00 | 1500 | 0.31 | 2.8 | |
| Loss due to SB | | 18 | 2.00 | | 0.14 | | 7 |

[a] Specific activity of cytochrome P-450 heme was 1960 dpm/nmol.
[b] PB-pretreated rats were given [4-$^{14}$C]δ-ALA (20 μCi) for 3 hr before sacrifice.
[c] $^{14}$C-cytochrome P-450 heme content was estimated by isolating the heme of microsomal CO-binding particles, which contain cytochrome P-450 as the only hemoprotein. Most (≃80%) of the radioactivity was found to be specifically associated with cytochrome P-450 heme. Specific activity of cytochrome P-450 heme (cpm/nmol) was 3900 (experiment 1) and 5000 (experiment 2).
[d] Loss of cytochrome P-450 determined spectrophotometrically.
[e] $^{14}$C radioactivity covalently bound to protein.
[f] Amount of $^{14}$C-heme-derived products covalently bound to microsomal protein.
[g] (*e*/*c*) × 100.

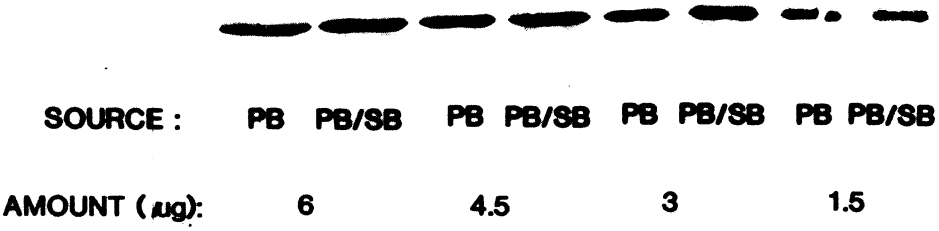


**Fig. 1.** Immunoblotting of cytochrome P-450$_b$ in liver microsomes from control (PB-pretreated) and SB-treated rats. Microsomes (1.5–6 μg of protein) were electrophoresed and the separated proteins were electrophoretically transferred to nitrocellulose membranes at 30 V overnight in 25 mM Tris (pH 8.3) containing 192 mM glycine and 20% (v/v) methanol. The sheets were then sequentially placed in 20 mM Tris, pH 7.5, containing 3% (w/v) gelatin, rabbit anti-rat P-450$_b$ immunoglobulin G, and goat anti-rabbit immunoglobin G horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) before being developed with horseradish peroxidase color reagent (Bio-Rad) as described previously (11).

scribed (15). On the other hand, when *in vitro* $^{14}$C-heme alkylation was monitored, hepatic microsomes were prepared from PB-pretreated rats given [$^{14}$C]δ-ALA and were incubated *in vitro* with NADPH (1.0 mM), SB (0.5 mM), and NADPH-generating system, in potassium phosphate buffer (0.1 M, pH 7.4) at a final concentration of 2 mg of protein/ml at 37° for 30 min. *In vitro* SB-mediated $^{14}$C-heme alkylation to microsomal protein was determined exactly as described (15). $^{14}$C-Cytochrome P-450 heme content was estimated by isolating the heme of microsomal CO-binding particles, which contain cytochrome P-450 as the only hemoprotein (17).

**SB-mediated P-450 heme *N*-alkylation.** Green pigments, the reporters of such an event, were isolated from SB-treated rat livers by procedures previously described (18) and were separated by two sequential thin layer chromatographic steps.

**SB-mediated inactivation of testosterone hydroxylase and ethoxycoumarin O-deethylase activities in purified reconstituted systems.** Purified P-450$_b$ and P-450 reductase, preincubated with DLPC, were incubated aerobically with unlabeled SB and NADPH at 37° as described above at concentrations and over time periods specified in each instance. At the end of the incubation, 40 μl of the assay mixtures were removed and used for either testosterone hydroxylase or ethoxycoumarin *O*-deethylase activities as follows. For assay of testosterone hydroxylase, the 40-μl aliquots were added to an incubation mixture (final volume, 0.5 ml) consisting of [$^{14}$C]testosterone (0.125 μCi, 230 μM), P-450 reductase (160 nM), DLPC (32 μM), isocitrate (5 mM), $MgCl_2$ (5 mM), NADPH (1 mM), and isocitrate dehydrogenase (0.25 units) in chelexed potassium phosphate buffer (0.1 M, pH 7.4) containing 2%, v/v, glycerol and 0.5 mM EDTA and were incubated at 37° for 10 min. Reactions were terminated and testosterone metabolites were assayed exactly as described previously, with authentic standards as carriers (11).

For the assay of ethoxycoumarin *O*-deethylase, the 40-μl aliquots were added to a reaction mixture (final volume, 1.0 ml) consisting of ethoxycoumarin (500 μM), P-450 reductase (80 nM), DLPC (32 μM), $MgCl_2$ (5 mM), and NADPH (300 μM) in chelexed potassium phosphate buffer (0.1 M, pH 7.4) containing 20%, v/v, glycerol and 20 μM EDTA and were then incubated at 37° for 10 min. Reactions were terminated and assayed exactly as described by Waxman and co-workers (19).

## Results and Discussion

Subcutaneous administration of SB to PB-pretreated rats results in loss of approximately 40% of the hepatic content of

TABLE 5

**Irreversible binding of [$^{14}$C]SB to rat liver microsomal protein after *in vitro* incubation**

| BSA[a] | P-450 loss[b] | Irreversibly bound $^{14}$C radioactivity | | [$^{14}$C]SB bound/P-450 inactivated | |
|---|---|---|---|---|---|
| | | | | Determination | Mean |
| | *nmol/incubation* | *cpm/incubation*[c] | *nmol of [$^{14}$C]SB*[d] | *nmol/nmol* | |
| − | 8.5 | 3985 | 3.2 | 0.38 | |
| | | | | | 0.45 |
| − | 6.5 | 4158 | 3.4 | 0.52 | |
| + | 8.4 | 5527 | 4.5 | 0.54 | |
| | | | | | 0.46 |
| + | 6.6 | 3046 | 2.5 | 0.38 | |

[a] Bovine serum albumin (BSA) (20 mg) was added to microsomal incubations (10 mg of protein). After incubation for 30 min, BSA was separated from the microsomal protein by centrifuging the incubation mixture at 100,000 × *g*. The microsomal pellet was used to determine the [$^{14}$C]SB-bound radioactivity.

[b] [$^{14}$C]SB (0.9 mM) was incubated with or without NADPH with liver microsomes prepared from PB-pretreated rats as described (Methods).

[c] $^{14}$C radioactivity covalently bound to protein.

[d] Specific activity of [$^{14}$C]SB was 1.23 × $10^3$ cpm/nmol.

TABLE 6

**SB-mediated inhibition of ethoxycoumarin *O*-deethylase activity in a cytochrome P-450$_b$-reconstituted system**

The complete system consists of cytochrome P-450$_b$, NADPH-cytochrome P-450 reductase (1.0 μM), DLPC (20 μg), EDTA (1.0 mM), glycerol (20%, v/v), and potassium phosphate buffer (100 mM, pH 7.4) in a final volume of 1.2 ml. Where appropriate, NADPH (1.0 mM), and/or SB (0.5 mM) were incubated at 37° for 10, 20, and 30 min, after which 40 μl of the mixture were removed and assayed in a fresh system (containing additional NADPH cytochrome P-450 reductase) for ethoxycoumarin *O*-deethylase activity (see Methods for details).

| Incubation time | System | Cytochrome P-450$_b$ | Ethoxycoumarin *O*-deethylase activity | |
|---|---|---|---|---|
| *min* | | *pmol/incubation* | *pmol of 7-OH-coumarin formed/min/incubation* | |
| 10 | −NADPH | 1224 | 1169 | |
| | −SB | 1080 | 1438 | |
| | Complete | 684 | 70 | |
| 20 | −NADPH | 1164 | 1193 | |
| | −SB | 936 | 1471 | |
| | Complete | 600 | 113 | |
| 30 | −NADPH | 1380 | 1934 | |
| | −SB | 1008 | 1498 | |
| | Complete | 516 | ND | |
| 30 | −NADPH | 2676 | 5757[a] | 6396[b] |
| | −SB | 2412 | 4809[a] | —[c] |
| | Complete[d] | 1266 | 78[a] | ND[e] |

[a] Aliquots of incubation mixture assayed directly as above.

[b] Aliquots of the reaction mixture after incubation were passed through G-25 Sephadex preequilibrated with the incubation buffer. Ethoxycoumarin *O*-deethylase activity was assayed after addition of fresh flavoprotein reductase (see Methods) as described above.

[c] Not determined.

[d] Incubation conditions were comparable to those above, except that incubations were carried out in a 3.0-ml volume.

[e] ND, none detected.

cytochrome P-450 within 1 hr of treatment (Table 1). Concomitantly, the hepatic activity of PROD, a functional marker for P-450$_b$, was reduced by 76%, whereas hepatic EM and BZP *N*-demethylase activities were reduced by 29% and 39%, respectively (Table 1). The observed discrepancy in the relative extent of inactivation of the three enzymes by SB may be explained by the fact that EM and BZP *N*-demethylase activities reflect functional contributions of several P-450 isozymes, including P-450$_b$. Together, these findings indicate that SB appears to largely spare isozymes other than P-450$_b$,

TABLE 7

**SB-mediated inhibition of testosterone hydroxylase activity in a cytochrome P-450$_b$-reconstituted system**

The complete system consists of cytochrome P-450$_b$, NADPH-cytochrome P-450 reductase (1.0 μM), DLPC (20 μg), EDTA (1.0 mM), glycerol (20%, v/v), and potassium phosphate buffer (100 mM, pH 7.4) in a final volume of 1.2 ml. Where indicated, NADPH (1.0 mM), and/or SB (0.5 mM) were incubated at 37° for 30 min, after which 40 μl of the mixture were removed and assayed in a fresh system (containing additional P-450 reductase) for testosterone hydroxylase activity (see Methods for details).

| System | Cytochrome P-450$_b$ | Testosterone hydroxylase activity | | |
|---|---|---|---|---|
| | | 16β | 16α | Androstenedione |
| | *nmol/incubation* | *nmol of hydroxytestosterone formed/min/incubation* | | |
| Trial 1 | | | | |
| −NADPH | 1.25 | 1.19 | 1.85 | 0.95 |
| −SB | 1.06 | 1.80 | 2.64 | 1.16 |
| Complete | 0.46 | 0 | 0 | 0 |
| Trial 2 | | | | |
| −NADPH | 1.36 | 1.85 | 2.95 | 1.72 |
| −SB | 1.16 | —[a] | — | — |
| Complete | 0.52 | 0 | 0 | 0 |

[a] Not determined.

TABLE 8

**Binding of [$^{14}$C]SB to cytochrome P-450$_b$ in a purified reconstituted system**

For experimental details see Methods. Values are mean ± standard deviation of at least three individual determinations.

| System | Cytochrome P-450$_b$ | Bound [$^{14}$C]SB[a] | | [$^{14}$C]SB/P-450$_b$ |
|---|---|---|---|---|
| | *nmol/ml* | *cpm* | *nmol of [$^{14}$C]SB/ml* | *nmol/nmol* |
| −NADPH | 1.85 ± 0.17 | 84.2 ± 32.8 | 0.07 ± 0.03 | 0.04 |
| −SB | 1.43 ± 0.07 | | | |
| Complete | 0.55 ± 0.11 | 658.0 ± 74.6 | 0.54 ± 0.06 | 0.98[b] (0.42)[c] |

[a] Values determined after passing the incubates through Sephadex G-25, which was found to remove loosely bound [$^{14}$C]SB and [$^{14}$C]SB-alkylated heme.

[b] [$^{14}$C]SB (nmol) irreversibly bound/nmol of intact P-450$_b$ remaining after incubation.

[c] nmol of [$^{14}$C]SB bound/nmol of spectrally detectable P-450$_b$ loss.

inasmuch as the observed reductions in hepatic P-450 content and functional activities can be accounted for largely by the specific hepatic content and functional contributions of P-450$_b$ to these activities. This notion is further supported by the finding that, in rats pretreated with the cytochrome P-450$_p$ inducer DEX, SB failed to appreciably lower hepatic cytochrome P-450 content and minimally affected P-450$_p$-dependent EM and erythromycin *N*-demethylase activities (Table 1). Furthermore, SB treatment of untreated rats failed to appreciably lower hepatic P-450 content and activity, indicating that constitutive isozymes (P-450$_h$ and P-450$_k$) among others were largely spared by the drug (Table 1).

Attempts to structurally and functionally repair SB-destroyed hepatic cytochrome P-450$_b$ by *in vitro* hemin incubation of rat liver homogenates resulted in an ≅17% increase in hepatic P-450 content,[2] while enhancing SB-inactivated EM and BZP *N*-demethylases from 71% to 90% and from 61% to 85%, respectively, but failed to significantly increase hepatic PROD activity (Table 1). Similarly administered equivalent doses of AIA, as expected, resulted in a relatively more pronounced inactivation of hepatic EM and BZP *N*-demethylases, as well as PROD (Table 2), consistent

[2] Increases have been expressed as percentage of original (pre-SB-inactivated) values of hepatic P-450 content and activity.

with the ability of this drug to inactivate multiple P-450 isozymes (11). However, hemin incubation was capable of restoring BZP *N*-demethylase activity from 33% to 86% and PROD activity from 21% to 105% after AIA-mediated inactivation, confirming our previous results with even higher doses (200 mg/kg) of AIA (11). These findings thus implied that P-450$_b$, although amenable to hemin-mediated repair after AIA inactivation, was refractory to it after SB-mediated inactivation. We find it intriguing that equivalent doses of aprobarbital, a 5-allyl-5-(1-isopropyl) analog of SB, resulted in 53% inactivation of PROD, which could be restored at least in part by exogenous hemin (Table 2). Such limited structure-activity comparisons indicate that hemin-irreparable inactivation of P-450$_b$ is unique to SB.

Furthermore, examination[3] of regioselective and stereoselective warfarin hydroxylations as functional markers for various hepatic P-450 isozymes confirmed that SB inactivated 50% of P-450$_b$-dependent (R)-4′-OH-warfarin hydroxylase rather selectively. Most other isozymes (P-450$_k$, (*S*)-7-OH-warfarin hydroxylase; P-450$_h$, (*S*)-4′-OH-warfarin hydroxylase) were minimally affected and of these SB-inactivated isozymes, apparently only P-450$_b$-dependent (*R*)-4′-OH-warfarin hydroxylase activity remained refractory to hemin-mediated repair. Its irreparability may account for the 23% of the total hepatic microsomal P-450 content (or 57% of the spectrally detectable SB-mediated P-450 loss) that is unavailable for hemin-mediated repair (Table 1).

The differential susceptibility of apo-P-450$_b$ for hemin-mediated repair after inactivation by AIA or SB may reflect several possible underlying causes. Thus, first, in the event of prosthetic heme alkylation by both agents, it is possible that the AIA-alkylated heme is removed more readily from the apocytochrome than its SB counterpart. Such tenacious residual binding of the green pigment (presumably at its active site) may prevent its replacement with fresh heme. Secondly, it is also possible that SB promotes prosthetic heme alkylation of the apocytochrome. Such an event has been reported to occur during inactivation of the major PB-inducible P-450 isozyme (presumably P-450$_b$) by several suicide inactivators, including AIA, norethindrone, fluroxene, and others (20–22). Although we failed to observe any appreciable heme-mediated alkylation of hepatic apocytochromes P-450 after *in vivo* administration of AIA (11), until verified, such a possibility could not be excluded in the case of SB. Thirdly, it is conceivable that SB-inactivation of P-450 enhances its proteolysis, thereby limiting the amounts of the apoprotein available for hemin repair. Finally, it is also possible that, unlike AIA, SB-mediated inactivation of cytochrome P-450$_b$ incurs alkylation of the apocytochrome as well as of the heme moiety. In this respect, SB-mediated inactivation of P-450$_b$ might be, at least in part, mechanistically similar to that observed with chloramphenicol (23, 24), and to those of cytochrome P-450$_c$ and cytochrome P-450$_\omega$ by 1-ethynylpyrene (25) and 10-undecynoic acid (26), respectively.

In an attempt to elucidate the mechanisms leading to the unusual refractoriness of SB-inactivated P-450$_b$ to hemin-mediated repair, we have examined each of the above possibilities. The likelihood that residual SB-derived green pigment could pose a significant deterrent to hemin-mediated reconstitution was examined by quantitating the microsomal levels of radiolabeled green pigments generated *in vivo* in SB-treated rats, after labeling the prosthetic heme moiety with [2,3-$^3$H]δ-ALA. As indicated (Table 3), $^3$H-green pigments isolated from hepatic microsomes of SB-treated rats accounted for 24% of SB-mediated microsomal cytochrome P-450 loss. Thus, if P-450$_b$ were indeed inactivated solely by SB-mediated heme alkylation, and if the green pigments persisted at its active site, then such residual binding could prevent up to 24% of the apohemoprotein molecules from accepting fresh heme and thereby from being reconstituted.

To determine whether SB-induced prosthetic heme alkylation of the apocytochrome could account for the remainder of the hemin-irreparable apoprotein, P-450 heme was labeled with [$^3$H]ALA in PB-pretreated rats. One half of the animals were then treated with SB; the other half served as controls. Quantitation of the irreversible $^3$H-heme binding in hepatic microsomes from such rats revealed that *in vivo* approximately 7% of the SB-mediated hepatic P-450 loss could be accounted for by irreversibly bound heme-protein adducts (Table 4). The relative extent of such binding could be slightly enhanced by *in vitro* incubation of liver microsomes containing prelabeled $^{14}$C-heme with SB, to 10% at best (Table 4). These results thus argue against irreversible heme binding as a significant deterrent to hemin reconstitution of cytochrome P-450$_b$.

Collectively, the above findings account for only 31% of the SB-inactivated cytochrome P-450 that is unavailable for structural and functional restoration, leaving nearly 69% of it unaccounted for. Furthermore, that proteolytic loss of apocytochrome P-450$_b$ (after its SB-mediated inactivation) was not responsible for such a finding was indicated by Western blotting of liver microsomal protein (Fig. 1). As can be seen at all amounts of microsomal protein blotted, no immunochemically detectable loss of apoP-450$_b$ is observed after SB treatment of rats for 1 hr when compared with microsomal apo-P-450$_b$ from corresponding non-SB-treated controls.

To examine whether alkylation of apocytochrome P-450$_b$ by a reactive intermediate generated during SB metabolism could account for the observed refractoriness of SB-inactivated cytochrome P-450$_b$ to hemin-mediated repair, preliminary experiments were conducted to determine whether, in fact, microsomal protein was found to be covalently labeled when rat liver microsomes were incubated aerobically with [$^{14}$C]SB in the presence of NADPH. Indeed, in these studies, an average of 0.45 nmol of [$^{14}$C]SB were bound per nmol of spectrally detectable P-450 lost from the incubation (Table 5), and this binding was absolutely dependent on catalytic participation of P-450$_b$ (not shown). In addition, if such binding were to be localized at the active site of the cytochrome, then it could fulfill an essential criterion for mechanism-based apocytochrome P-450$_b$ alkylation by the drug. Such a possibility was strengthened by the finding that inclusion of bovine serum albumin (20 mg) in the incubation mixture failed to appreciably attenuate [$^{14}$C]SB binding to liver microsomal protein (Table 5), a result expected if a very reactive [$^{14}$C]SB metabolite incapable of escaping from the active site were responsible for the irreversible binding.

To examine this possibility more directly, SB-mediated inactivation of purified P-450$_b$ was examined after its reconstitution with purified NADPH-cytochrome P-450

[3] J. M. Lunetta, A. E. Rettie, and M. A. Correia, preliminary findings.

reductase. In such systems, SB caused a time- and NADPH-dependent loss of purified P-450$_b$ detectable spectrally as well as functionally by its ethoxycoumarin *O*-deethylase activity (Table 6) and of its testosterone 16$\alpha$- and 16$\beta$-hydroxylase and 17-dehydrogenase (androstenedione formation) activities (Table 7). Moreover, no appreciable functional activity could be detected, although as much as 50% of the P-450 chromophore remained. Such functional loss is not due to loss of P-450 reductase activity because additional (fresh) reductase was routinely included before reassay of functional activity. Furthermore, that the loss was not due to persistence of a SB-derived inhibitor at the active site of P-450 was shown by passing aliquots of the SB-incubated complete reaction mixture through Sephadex G-25 to remove any such residual inhibitors before functional assay. No recovery of functional activity was observed in such gel-filtrated aliquots of the reaction mixture, indicating irreversible loss of P-450$_b$ function after SB-mediated inactivation (Table 6).

When [$^{14}$C]SB was included in the reconstituted system, NADPH-dependent [$^{14}$C]SB binding to P-450 was observed to the extent of 0.42 nmol of SB bound/nmol of P-450$_b$ chromophore loss after passage of incubates through Sephadex G-25, which is known to remove loosely bound [$^{14}$C]SB and $^{14}$C-alkylated heme derivatives. This value is comparable to that observed in intact rat liver microsomes, underscoring once again the relative selectivity of SB inactivation for P-450$_b$. Moreover, if this binding is expressed as nmol of SB bound/nmol of P-450$_b$ remaining after complete inactivation, a value of 0.98 is obtained, which suggests that the binding is nearly equimolar to the functionally inactive residual chromophore (Table 8).

Selectivity of [$^{14}$C]SB binding to P-450$_b$ was determined by radioquantitation of Western blotted proteins after their incubation and SDS-PAGE separation as described (Methods). Only P-450$_b$ from NADPH-supplemented incubations exhibited significant [$^{14}$C]SB radioactivity. No significant counts were associated with the flavoprotein reductase from the same incubation system. Furthermore, precipitation and exhaustive $H_2SO_4$/methanol washes of the incubated proteins allowed us to confirm that such [$^{14}$C]SB binding to P-450$_b$ was irreversible (Table 8).

Collectively, the findings with isozyme-selective functional markers strongly argue that SB is highly selective for inactivation of P-450$_b$, thereby confirming similar results from more limited isozyme comparisons (12). The relative selectivity of SB for inactivation of P-450$_b$ both *in vivo* and *in vitro*, the significant formation of green pigments in parallel, and the consistent observation of NADPH-dependent [$^{14}$C]SB alkylation of the apo-P-450$_b$[4] together suggest that SB-mediated inactivation of this particular isozyme must entail partitioning between heme alkylation, apoprotein alkylation, and SB oxidation. In otherwise untreated rats given SB, the major oxidized SB metabolites in the urine appear to be the two diastereoisomeric 5-allyl-5-(3′-hydroxy-1′-methylbutyl) barbituric acids, followed by secodiol [the hydrolyzed product of the SB-epoxide 5-(2′,3′-oxypropyl)-5-(1′-methylbutyl) barbituric acid], the second largest urinary metabolite (27). These findings thus suggest that, in untreated rats, hydroxylation of the methylbutyl side chain is the predominant reaction. However, in contrast to the oxidative activation of the allyl side chain, oxidation of methylbutyl side chains in barbiturates does not appear to result in P-450 destruction (3, 4). Although it is presently unknown whether PB induction results in stimulation of SB epoxidation, it is likely that the increased destruction of P-450$_b$ observed on induction might be due to such an event.

However, extensive direct and indirect evidence on the mechanism of P-450 heme *N*-alkylation by olefinic substrates tend to exonerate the epoxides as formal intermediates in P-450 destruction (8, 28). P-450 heme *N*-alkylation appears to intervene before the epoxide is formed (8). Because SB is formally an end-substituted olefin and its olefinic moiety appears to be critical for P-450$_b$ destruction, in analogy to other olefinic suicide substrates, it is reasonable to speculate that the olefinic $\pi$-bond is similarly activated by P-450 to a cation radical, which subsequently alkylates the pyrrolic heme nitrogen at its terminal carbon, while incurring *O*-addition at its internal carbon. Furthermore, if the analogy can be extended further, such additions would appear to occur on the *re*-face of the olefin, a possibility to be confirmed by structural characterization of the SB-alkylated heme adduct. On the other hand, epoxide formation can occur on either face of certain olefins, resulting in two enantiomers (29). Because a consistent fraction of P-450$_b$ apoprotein also undergoes enzyme-dependent alkylation, apparently at the active site, it remains to be determined whether such alkylation involves the cation-radical species and occurs on the terminal or internal carbon and whether it incurs a *si*-face or *re*-face attack of the SB allylic $\pi$-bond. In such an event, P-450-mediated oxidation of SB would appear to partition between destructive events (prosthetic heme alkylation and apoprotein alkylation) and epoxide formation.[5] Our finding that SB fully inactivates purified P-450$_b$ functionally with partial retention of its spectral chromophore is thus consistent with such a scheme. Functional inactivation via apoprotein alkylation of P-450$_b$, P-450$_c$, and P-450$_\omega$ by chloramphenicol, *l*-ethynylpyrene, and 10-undecynoic acid, respectively (23–26), is also known to occur without loss of the isozyme chromophore. However, chloramphenicol-mediated apo-P-450$_b$ alkylation appears to render P-450$_b$ functionally inactive by impairing P-450 reductase/P-450 electron transfer (31).

Our findings thus indicate that SB-mediated heme alkylation and apoprotein alkylation are largely responsible for P-450$_b$ inactivation during its oxidative metabolism of SB, whereas irreversible heme binding may contribute to it in a minor way. Such a scenario accounts for the failure of hemin to fully restore P-450$_b$ function after SB-mediated inactivation but does not explain why it fails to structurally repair the enzyme at least in part. In theory, given that very little of the *N*-alkyl adduct remains associated with the membrane, the heme-*N*-alkylated P-450 fraction should have been amenable to such repair. Such resistance to repair is particularly difficult to reconcile with the relative ease with which P-450$_b$ is repaired after its AIA-mediated heme N-alkylation (Table 2) (11), even in the presence of much higher levels of membrane-bound adducts. It is possible of course that the SB-heme adduct, being more

[4] Similar findings were obtained by Walsh and co-workers in preliminary experiments (C. T. Walsh, personal communication).

[5] A partition ratio of 16 for heme alkylation and epoxide formation has in fact been reported for P-450-dependent SB metabolism (30).

lipophilic, is retained more firmly at the active site, whereas that of AIA, being relatively less so, is extruded to the exterior, thereby permitting greater access of fresh heme into the active site.

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