Biochemical Pharmacology, Vol. 38, No. 22, pp. 4103–4113, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

A MODEL OF CYTOCHROME P-450-CENTERED HEPATIC DYSFUNCTION IN DRUG METABOLISM INDUCED BY COBALT-PROTOPORPHYRIN ADMINISTRATION*

BARRY B. MUHOBERAC,†‡ TERUKI HANEW,§ SUSAN HALTER‖ and STEVEN SCHENKER§

‡ Department of Chemistry, Purdue University School of Science, Indiana University-Purdue University at Indianapolis, Indianapolis, IN 46205; § Department of Medicine, Division of Gastroenterology and Nutrition, The University of Texas Health Science Center, San Antonio, TX 78284, and Audie L. Murphy Memorial Veterans Hospital, San Antonio, TX 78284; and ‖ Department of Pathology, Vanderbilt University, Nashville, TN 37203, and Veterans Administration Medical Center, Nashville, TN 37203, U.S.A.

(*Received* 13 *May* 1988; *accepted* 28 *April* 1989)

Abstract—Cobalt-protoporphyrin treatment disrupts cytochrome P-450-centered drug metabolism and is known to decrease significantly the cytochrome P-450 content of the liver. This study assesses further the correlations between biochemical and functional changes induced by Co-protoporphyrin. Specifically, it confirmed the fall in cytochrome P-450 levels in liver and demonstrated that both NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase activities decreased in a dose-dependent manner, albeit to a lesser degree, upon Co-protoporphyrin administration. Furthermore, plasma clearance of the marker drug aminopyrine fell off abruptly with a minimal decrease in cytochrome P-450 content, and then monotonically with its further depletion. Both aminopyrine and caffeine demethylation, as measured by the amount of radiolabeled CO_2 exhaled, also decreased with diminishing cytochrome P-450 content. With aminopyrine the decrease was abrupt but with caffeine biphasic, consistent with preferential isozyme depletion. The drop in oxidative drug metabolism measured by these two *in vivo* techniques occurred in the absence of organellar damage to hepatocytes, as observed by electron microscopy. *In vitro* studies of aminopyrine metabolism in microsomes prepared from rats with and without Co-protoporphyrin injection proved to be consistent with the *in vivo* studies. Moreover aminopyrine V_{max} decreased and K_m increased with decreasing cytochrome P-450 content, suggesting preferential isozyme depletion. Furthermore, the changes in aminopyrine intrinsic clearance predicted by the *in vitro* V_{max} and K_m values agreed with those measured by *in vivo* plasma clearance. Taken together, these data suggest that Co-protoporphyrin treatment can be used to produce a model of altered cytochrome P-450-centered drug metabolism, as measured consistently by several techniques. However, this model appears to be more complex than one involving nonspecific depletion of cytochrome P-450 alone, and may be influenced also by concomitant changes in the electron transport chain or other aspects of hepatic metabolism.

Cytochrome P-450 is the site of metabolism of many drugs. This biotransformation can be critical to drug elimination, although alteration to more toxic metabolites may also occur. Drug modification is dependent on the transfer of electrons from NADPH (and NADH) through NADPH–cytochrome P-450 reductase (and NADH–cytochrome b_5 reductase/cytochrome b_5) to cytochrome P-450 as well as on the transport and binding of the drug to cytochrome P-450. Indeed, cytochrome P-450-centered drug metabolism involves a series of steps, depending not only on cytochrome P-450, but also on the presence and redox state of members of the electron transport chain. The redox state of the electron transport chain, in turn, depends significantly on the availability of hepatic reducing equivalents.

The effects of inorganic Co^{2+} on the liver have been studied extensively [1–5]. Subcutaneous injections cause a decrease in microsomal cytochrome P-450 and heme content as well as a decrease in N-demethylation of ethylmorphine. Furthermore, there is an increase in heme oxygenase activity and an initial inhibition followed by a rebound in δ-aminolevulinate synthase activities. The former is the rate-limiting enzyme in hepatic heme degradation and the latter the rate-limiting enzyme in heme sythesis. These changes occur rapidly after Co^{2+} injection; however, the above-mentioned parameters return to normal within 72 hr. Furthermore, the dose of Co^{2+} needed to induce significant changes in the cytochrome P-450 content has been reported to produce no apparent change in the NADPH–cytochrome P-450 reductase activity [2, 4].

The effects of inorganic Co^{2+} are less substantial and protracted than those produced by Co-proto¶ injections [6, 7]. One dose of Co-proto (125 μmol/kg) can decrease microsomal cytochrome P-450 content to approximately 25% of normal where it remains for about 10 days. The microsomal heme content and *N*-demethylase activity drop to about 50 and 10% of control respectively. The heme oxy-

* This work was supported by NIAAA grants R23 AA06935 and R01 AA05814.

† Corresponding author: Barry B. Muhoberac, Ph.D., Department of Chemistry, Purdue University School of Science, Indiana University-Purdue University at Indianapolis, 1125 East 38th St., Indianapolis, IN 46205.

¶ Abbreviations: Co-proto, cobalt(III) protoporphyrin; and heme, iron protoporphyrin.

genase activity is enhanced but, in contrast to inorganic Co^{2+}, the δ-aminolevulinate synthase activity is decreased. This combination, along with the inability of the heme oxygenase system to metabolize Co-proto [8], is probably related to the severity of the cytochrome P-450 depletion. This severe, prolonged depletion has led Drummond and Kappas [6] to suggest that the Co-proto-treated animal can provide a valuable model for examining cytochrome P-450-dependent drug metabolism.

This study addresses more extensively the potential use of the Co-proto-treated rat as a model of altered cytochrome P-450-centered drug metabolism by correlating liver cytochrome P-450 levels and other aspects of cytochrome P-450 function with well-established measurements of hepatic function. The techniques used were: (1) aminopyrine plasma clearance, (2) the aminopyrine and caffeine "breath tests," which noninvasively quantitate drug demethylation of these substrates through CO_2 exhaled, and (3) *in vitro* enzyme kinetics of aminopyrine demethylation through formaldehyde formation. These different measurements complement each other and give a more nearly complete picture of the effects of Co-proto on drug modification and elimination.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats of approximately 275 g were purchased from Harlan Sprague–Dawley Inc., Indianapolis, IN. The animals were fed standard Purina 5015 rat chow diet and allowed at least 1 week for acclimation to local conditions.

Co-proto preparation and injections. Co-proto (Porphyrin Products, Logan, UT) solutions were prepared by dissolving the reagent in 0.1 N NaOH, then titrating to neutral pH with 0.1 N HCl, and lastly bringing to volume with 0.9% NaCl. These solutions were prepared immediately before injection. Rats were injected subcutaneously with doses ranging downward from 60 μmol/kg rat weight, and the control animals were injected with an equivalent volume of 0.9% NaCl. Drug elimination and enzyme activity studies were performed 72 hr after the single dose of Co-proto. Food and water were provided *ad lib.* until 12 hr before each study, and at this time food was removed.

Plasma clearance and aminopyrine quantification. Animals were prepared for plasma clearance determination 24 hr after Co-proto or NaCl injections by catheterization of the jugular vein with PE 50 tubing under ether anesthetic. The tubing exited at the back of the neck and was heparinized to maintain patency. The animals were allowed 48 hr to recover before beginning plasma clearance studies through administration by catheter of a 50 mg aminopyrine/ml 0.9% NaCl solution at 30 mg/kg rat weight. This delayed procedure was used to avoid any ether-induced effects on drug clearance [9]. Blood was collected serially from the tail vein in 10 equally timed samples, each of 150 μl, over a period of 150 min. The samples were made basic by addition of sodium borate, extracted into an organic mixture (95% chloroform/5% propanol), and evaporated to dryness. The residue was dissolved in the mobile phase (10% acetonitrile/4% acetic acid/86% H_2O), and the aminopyrine concentration was determined by HPLC (Waters Associates, Milford, MA) using a μ Bondapak C18 column at a flow rate of 2 ml/min. The chromatography was performed isocratically with the elutant monitored at 254 nm using a Waters 440 absorbance detector. Samples were area-ratio integrated to determine aminopyrine concentration.

The time course of the removal of aminopyrine from plasma was analyzed by first determining the elimination half-life, $t_{1/2}$ (β), from the latter (0.2 to 3 hr) portion of the data by linear regression of the logarithm of the plasma concentration versus time curve. The aminopyrine plasma clearance (Cl) was obtained using the trapezoidal rule where Cl = dose/area under plasma concentration curve extrapolated to infinity. The volume of distribution, V_d, was calculated as Cl/β.

Aminopyrine and caffeine breath test with $^{14}CO_2$ quantification. Radiolabeled aminopyrine (Amersham, Arlington Heights, IL) was injected intraperitoneally in 0.9% NaCl (sp. act. 250 μCi/140 ml) at 0.25 ml/200 g rat weight. Rats were housed individually in airtight restraining cages. Exhaled $^{14}CO_2$ was trapped by first drawing it through concentrated H_2SO_4 to remove moisture and then through a scintillation vial containing 10 ml of 67% methanol/33% ethanolamine solution. Ten milliliters of ACS (Amersham) was added to each vial before quantification using a Beckman LS 6800 liquid scintillation counter. The automatic external standardization procedure was used to correct for quenching, and 10 μl of the radiolabeled NaCl solution was used as standard. The amount of eliminated $^{14}CO_2$ was expressed as the cumulative percent of the injected dose. Radiolabeled caffeine in 0.9% NaCl (sp. act. 250 μCi/100 ml) was injected at 0.25 ml/200 g rat weight. Exhaled $^{14}CO_2$ was trapped and quantitated as with the radiolabeled aminopyrine. Caffeine was labeled at the 1 position and aminopyrine at both methyl groups at the 5 position.

Preparation of microsomes. Animals were killed by decapitation without the use of anesthetics. Livers were removed and perfused with 50 ml of cold 0.9% NaCl via portal and hepatic veins until complete blanching occurred. The liver was minced and then homogenized in 5 vol. of 250 mM sucrose/g liver weight using a glass/teflon homogenizer. The homogenate was centrifuged at 8,000 g for 15 min and the supernatant fraction at 19,000 g for 15 min. The microsomal pellet was then isolated by ultracentrifugation at 105,000 g for 60 min. The supernatant fraction and lipid were separated from the pellet, which was resuspended by hand using a glass/teflon homogenizer in 50 mM Tris–HCl, pH 7.7 (at 25°). The protein concentration was determined by the method of Lowry *et al.* [10] using bovine serum albumin as standard.

Microsomal enzyme content and assays. The microsomal contents of cytochrome P-450 and cytochrome b_5 were determined by optical difference spectroscopy using an Aminco-Chance DW2 spectrophotometer in split beam mode. The content of cytochrome b_5 was determined using NADH-reduced microsomes and $\Delta\varepsilon = 185/\text{mM/cm}$ as described by Estabrook and Werringloer [11]. After

obtaining the cytochrome b_5 spectrum, microsomes from both cuvettes were mixed, bubbled with CO for 30 sec, and again divided into two cuvettes. After obtaining a baseline, dithionite was added to the sample cuvette and from the difference spectrum the cytochrome P-450 content was calculated using $\Delta\varepsilon$ (510 nm–450 nm) = 100/mM/cm.

The aminopyrine *N*-demethylase activity was determined according to the method of Litterst *et al.* [12] with the slight modifications described below. The formaldehyde produced was quantified by the method of Nash [13]. Briefly, demethylation was initiated by adding microsomes (1–3 mg/ml) to a reaction mixture containing aminopyrine varied from 0.66 to 25.0 mM, the regenerating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, 5 mM $MgCl_2$, and 2 units of glucose-6-phosphate dehydrogenase), and 100 mM Tris–HCl, pH 7.4 (at 37°), to a final volume of 3.0 ml. Glucose-6-phosphate dehydrogenase and its substrate were obtained from the Sigma Chemical Co., St. Louis, MO. The reaction mixture was incubated at 37° in a shaking water bath, and formaldehyde formation was found to be linear at low and high aminopyrine concentrations in both Co-proto and untreated animals for at least 20 min. Formaldehyde formation was stopped with 10% trichloroacetic acid, and the solution was centrifuged. The formaldehyde concentration of the supernatant fraction was quantitated with addition of acetylacetone and ammonia. Standard curves with formaldehyde stock were produced for each set of animals. The values of maximum velocity (V_{max}) and the apparent Michaelis constant (K_m) were determined first through Hanes plots, and then with greater accuracy through a modified interactive nonlinear regression computer program with proportional weighing of the standard deviation [14].

NADPH–cytochrome P-450 reductase (NADPH–cytochrome *c* reductase) activity was measured as described by Williams and Kamin [15] with addition of NADPH by following the increase at 550 nm of cytochrome *c* with $\Delta\varepsilon = 21$/mM/cm. A similar procedure was followed with NADH to quantitate NADH–cytochrome b_5 reductase activity. The cytochrome *c* and substrates were purchased from the Sigma Chemical Co.

Electron microscopy. Rat liver slices from control and Co-proto-treated animals were fixed in 3% glutaraldehyde in 100 mM cacodylate buffer. Tissue was post-fixed in osmium tetroxide, inbloc stained with uranyl acetate, dehydrated in ethanol, and embedded in Spurr (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with lead citrate and uranyl acetate, and were viewed in two sets of animals in a coded (blinded) fashion with a Hitachi H-600 transmission electron microscope.

Statistical analysis. Measurements are expressed as the mean ± the standard error of the mean (SE). Student's *t*-test was used to compare differences in means, and a $P < 0.05$ (two-tailed) was accepted as significant.

RESULTS

Enzyme content and activity. Figure 1A shows the dose-dependent decrease in microsomal cytochrome P-450 content at 72 hr after a single Co-proto injection. The rate of decrease with dose was greater at lower Co-proto doses than with larger doses. Table 1 gives the mean decrease at doses of 15, 30 and 60 μmol/kg rat weight with the appropriate statistical analyses. The cytochrome P-450 content at the highest dose was decreased to approximately 30% of that found with control animals in agreement with the report by Drummond and Kappas [6], i.e. 25% of control values with a Co-proto dose of 125 μmol/kg. In the present study a dose greater than 60 μmol/kg rat weight caused pronounced debility and occasional death, and little additional decrease in cytochrome P-450 content was achieved with the higher dose. There was no consistent, significant change in total liver microsomal protein with Co-proto administration. The cytochrome b_5 content (Fig. 1B, Table 1) fell to approximately 70% of control which is significantly less than the decrease in cytochrome P-450 and in line with that reported previously (66% at 125 μmol/kg dose) [6].

Both the NADPH–cytochrome P-450 reductase (Fig. 1C) and NADPH–cytochrome b_5 reductase (Fig. 1D) activities fell in a dose-dependent manner in response to Co-proto treatment. Injection of 60 μmol/kg rat weight decreased these activities to approximately 45 and 50% of control values respectively (Table 1). The NADPH–cytochrome P-450 reductase activity remaining is in line with the 44% reported by Kirkun and Cederbaum [16] but is greater than the 22% of Cheeseman *et al.* [17]. As with cytochrome P-450, a greater rate of decrease with dose occurred at lower Co-proto doses than with larger doses.

Plasma aminopyrine elimination. The plasma elimination of the marker drug aminopyrine was suppressed markedly by Co-proto injections. Table 2 shows that at a dose of 60 μmol/kg rat weight the plasma clearance (*Cl*) decreased to 21% of control and half-life ($t_{1/2}$) increased proportionally, i.e. about 4-fold. As one would expect for that ratio, there was no statistically significant change in the volume of distribution (V_d). Figure 2 expresses the aminopyrine plasma clearance as a function of decrease in cytochrome P-450 content. It shows a large decrease in clearance (536 to about 300 ml/hr) with only a 10% (or less) decrease in cytochrome P-450 content followed by a linear clearance decrease as cytochrome P-450 content fell from 90 to 20% of control. The extrapolated clearance fell approximately to zero at a projected total absence of cytochrome P-450.

Aminopyrine and caffeine breath tests. The cumulative $^{14}CO_2$ exhaled (after administration of trace doses of radiolabeled aminopyrine or caffeine) was depressed significantly after Co-proto injections. Figure 3A shows the abrupt decrease in cumulative $^{14}CO_2$ exhaled through 45 min from the marker drug aminopyrine, expressed as percent of control, as a function of decreasing cytochrome P-450 content. Approximately 17% of the injected dose was exhaled from the control animals over this period of time, and the cumulative $^{14}CO_2$ exhaled versus time for both controls and treated animals remained linear (data not shown). For the marker drug caffeine, Fig.

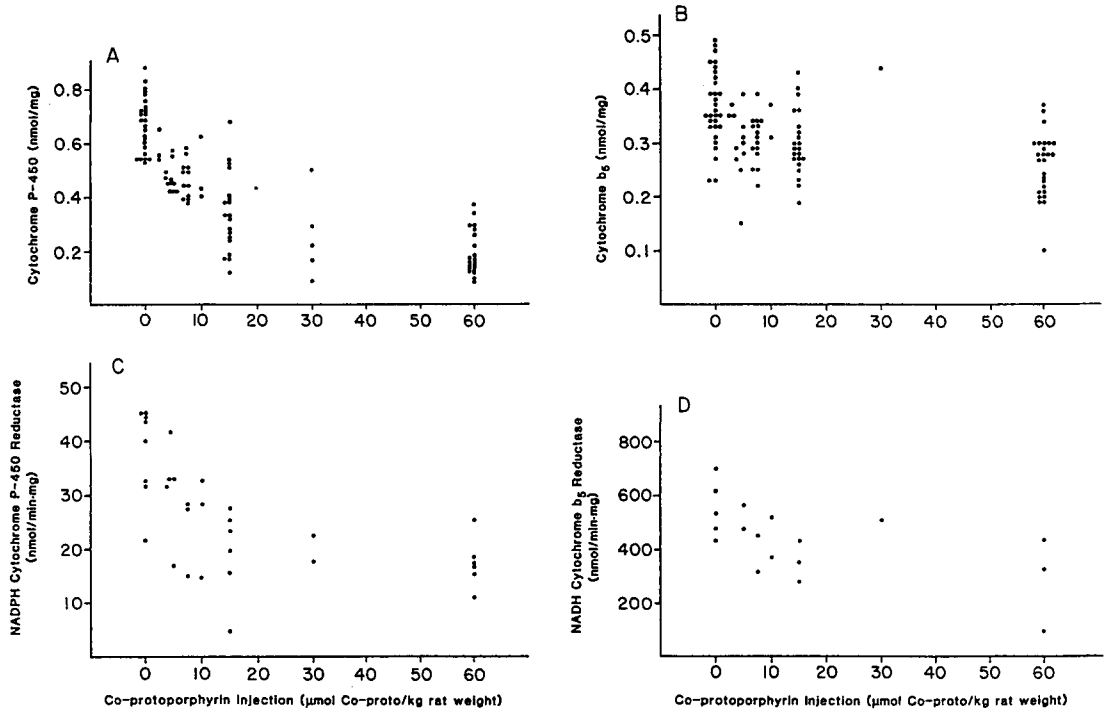

Fig. 1. Effect of a single Co-proto injection on cytochrome P-450 content (A), cytochrome b_5 content (B), NADPH–cytochrome P-450 reductase activity (C), and NADH–cytochrome b_5 reductase activity (D) in liver microsomes. Animals were injected with various amounts of Co-proto 72 hr prior to being killed, and each point represents one animal.

Table 1. Decrease in enzyme content and activity after cobalt-protoporphyrin (Co-proto) administration

Co-proto injection (μmol/kg rat weight)	Cytochrome P-450 (nmol/mg microsomal protein)	Cytochrome b_5 (nmol/mg microsomal protein)	NADPH–Cytochrome P-450 reductase (nmol/min·mg microsomal protein)	NADH–Cytochrome b_5 reductase (nmol/min·mg microsomal protein)
0	0.671 ± 0.020 (25)	0.367 ± 0.013 (30)	37.9 ± 3.0 (8)	549 ± 48 (5)
15	0.340 ± 0.034* (19)	0.300 ± 0.013* (22)	19.4 ± 3.4† (6)	335 ± 43† (3)
30	0.254 ± 0.070* (5)		20.0 ± 2.5† (2)	
60	0.192 ± 0.018* (21)	0.259 ± 0.012* (26)	17.6 ± 1.9* (6)	287 ± 99† (3)

Values are means ± SE, with the number of animals per group given in parentheses.
* $P < 0.01$.
† $P < 0.05$.

Table 2. Aminopyrine clearance after cobalt-protoporphyrin (Co-proto) administration

	Cl (ml/hr)	$t_{1/2}$ (min)	V_d (ml/kg)
Control (8)	536 ± 33	18.9 ± 0.7	797 ± 50
Co-proto dose of 60 μmol/kg rat weight (6)	114 ± 20*	79.2 ± 7.2*	646 ± 67†

Values are means ± SE, with the number of animals per group given in parentheses.
* $P < 0.001$.
† $P = 0.090$.

3B shows that the decrease in cumulative $^{14}CO_2$ exhaled through 45 min was more gradual with non-linearity. In the controls only about 1.6% of the injected caffeine was exhaled over this period of time, and the cumulative $^{14}CO_2$ exhaled versus time remained linear for all animals up until 150 min.

Because of the small amount of $^{14}CO_2$ evolved from caffeine over 45 min, the cumulative $^{14}CO_2$ exhaled over 150 min was also quantitated, as given in Fig. 3C. This response was more clearly biphasic and distinct from that of aminopyrine. Only 7.2% of the injected caffeine was exhaled over 150 min. The cumulative $^{14}CO_2$ exhaled through 150 min from aminopyrine demethylation was not included in Fig.

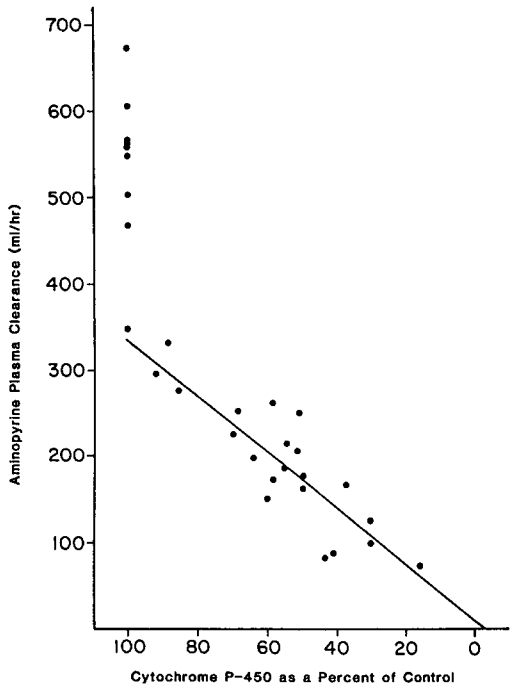

Fig. 2. Correlation between aminopyrine plasma clearance and Co-proto-induced decrease in microsomal cytochrome P-450 content.

3 because the cumulative $^{14}CO_2$ exhaled versus time was nonlinear after 45 min. An average of 32% of the injected dose of aminopyrine was exhaled through 150 min in the controls, and this may correspond to as much as 64% of the injected aminopyrine being metabolized since the amount of radiolabeled carbon lost to other metabolic pathways can be as much as 50% [18]. This severe substrate depletion likely causes deviation from linearity and makes the shape of the cumulative $^{14}CO_2$ versus cytochrome P-450 depletion curve dependent upon the time of $^{14}CO_2$ accumulation for times greater than 45 min. Along these lines, a depressed rate of transfer of labeled carbon between metabolic pools could potentially depress $^{14}CO_2$ evolution independent of demethylation by cytochrome P-450. However, no consistent, statistically significant alteration of $^{14}CO_2$ evolution from an injection of radiolabeled formate was observed following Co-proto treatment sufficient to depress cytochrome P-450 markedly, and hence no adjustment for this was included.

Kinetics of in vitro *aminopyrine metabolism.* Panels A and B of Fig. 4 show that the V_{max} and K_m of aminopyrine demethylation by liver microsomes were altered by prior *in vivo* Co-proto treatment. There was a monotonic decrease in V_{max} from 4.9 nmol/min·mg at 100% cytochrome P-450 content to 1.3 nmol/min·mg at 20%, a decrease to 27% of control. The variation in V_{max} lessened with from 60 to 20% of the control level of cytochrome P-450 remaining, and the extrapolated decrease in V_{max} approximated zero at a projected total absence of the enzyme. Although not as clearly monotonic as the V_{max}, the K_m increased from 1.1 to 3.1 mM over

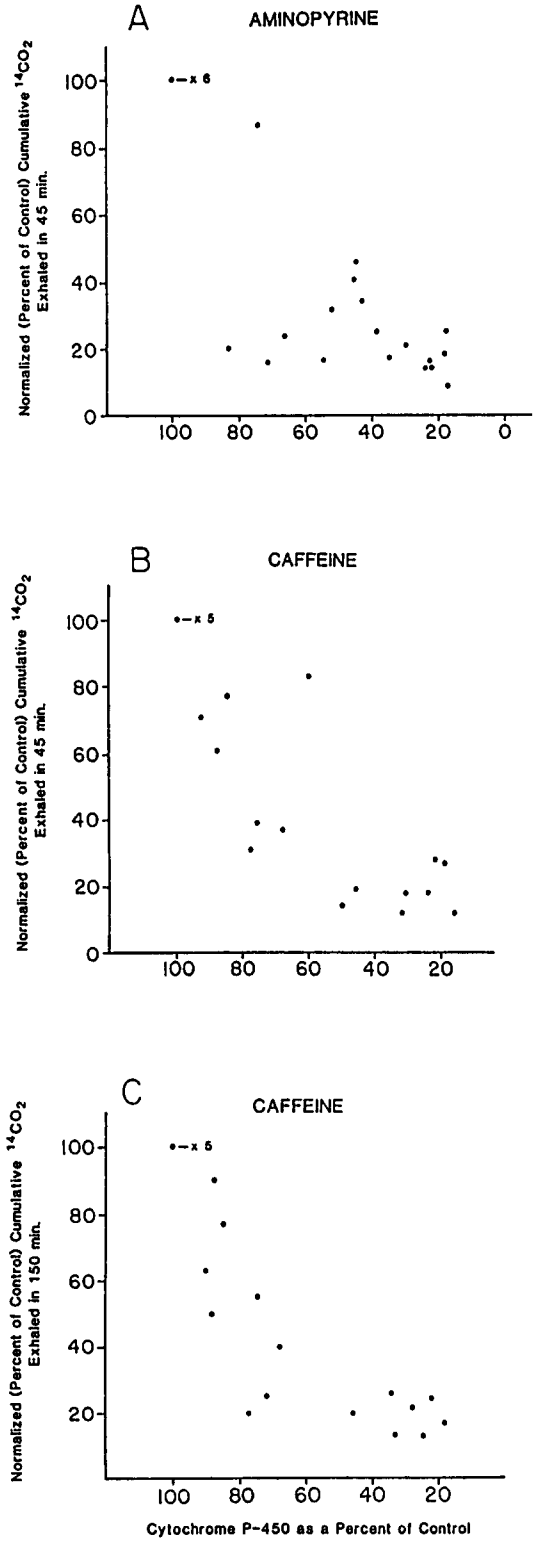

Fig. 3. Correlation between cumulative $^{14}CO_2$ exhaled (demethylation) and Co-proto-induced decrease in microsomal cytochrome P-450 content. Radiolabeled aminopyrine (A) and caffeine (B) were injected intraperitoneally, and the cumulative amount of $^{14}CO_2$ exhaled in 45 min was quantitated by liquid scintillation and expressed as percent of control. Radiolabeled caffeine (C) was also quantitated 150 min post injection.

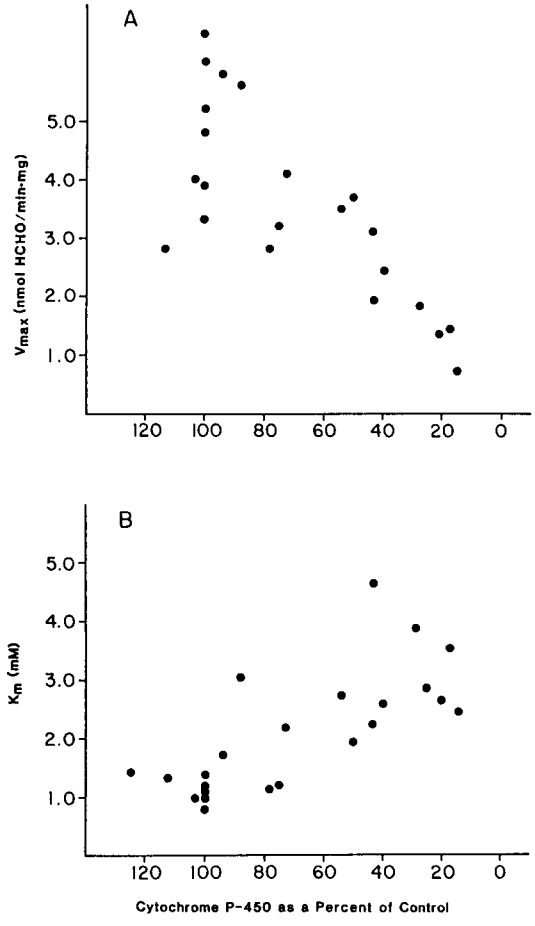

Fig. 4. Relationship of the V_{max} (A) and K_m (B) of aminopyrine N-demethylation to Co-proto-induced decrease in microsomal cytochrome P-450 content.

the decrease in cytochrome P-450 content. This was a K_m increase of about 270%.

Electron microscopy of liver tissues. Because drug elimination could be impaired significantly by tissue damage instead of depletion of a particularly enzyme or enzymes, tissue integrity was assessed by electron and light microscopy. No difference between liver sections from these animals was found, as seen in Fig. 5 (top and bottom panels), although a morphometric analysis was not carried out. Specifically, the smooth endoplasmic reticulum and mitochondria showed no evidence of damage, implying that the tissue was viable and not hypoxic. Similarly, no morphological changes were observed by light microscopy.

DISCUSSION

A more detailed understanding of depressed oxidative drug metabolism, whether caused by liver disease, reversible inhibition of cytochrome P-450 by drugs (i.e. cimetidine), or destruction of cytochrome P-450 by hepatotoxins, is clearly important. Prospects for this were enhanced when Drummond and Kappas [6], using a series of *in vitro* measurements, proposed the Co-proto-treated rat as a model for cytochrome P-450-centered hepatic dysfunction. The present study expands our understanding of the model in four ways. First, it *extends* the model of Drummond and Kappas from the level of *in vitro* enzyme assays to that of the whole animal by quantifying important Co-proto-induced changes in *in vivo* drug elimination measured by plasma clearance and the breath test. Second, it *broadens* the model by showing dose-dependent *in vitro* changes in electron donor activities crucial to the mixed-function oxidase system as well as *in vitro* changes in cytochrome P-450 enzyme kinetics. Third, this study *correlates* Co-proto-induced changes at the *in vitro* level with those observed *in vivo*. Finally, it *evaluates* critically the potential use of the Co-proto-treated rat as a model for cytochrome P-450-centered hepatic dysfunction.

The most pharmacologically important measure of drug elimination, the *in vivo* plasma clearance, was reduced by Co-proto treatment. The clearance of the marker drug aminopyrine, after an initial abrupt drop, decreased linearly with depletion of hepatic cytochrome P-450 and extrapolated approximately to zero clearance at total absence of the enzyme. The linear extrapolation is important because it shows that elimination does not terminate prior to cytochrome P-450 depletion and implies that no residual aminopyrine elimination should remain after such depletion. Early termination would suggest that cytochrome P-450 content was not a limiting factor in the model, and late termination could involve other pathways of aminopyrine elimination. Either factor would limit the usefulness of the model, which implies after the initial drop a 1 : 1 relationship between cytochrome P-450 destruction and *in vivo* inability to remove aminopyrine. This interpretation refers to total cytochrome P-450 at this point and not to its isozymes which may have differential affinity for the drug.

The initial 40% drop in aminopyrine plasma clearance with only 10% cytochrome P-450 depletion is puzzling and may be due to several effects. Co-proto treatment may preferentially and potently deplete an isozyme(s) of cytochrome P-450 that accounts for a large percent of aminopyrine demethylation. Alternatively metabolic factors outside the mixed-function oxidase system, such as the redox state of the liver, could be changed significantly by Co-proto and cause depressed oxidative drug metabolism [19, 20]. Also serum testosterone, thyroxine and 3,5,3′-triiodothyronine levels are decreased by Co-proto treatment without reciprocal elevations in either serum lutinizing hormone or thyroid-stimulating hormone [21, 22]. These changes imply endocrine alterations and suggest secondary effects on drug metabolism. Finally, although less likely with the low extraction ratio drug aminopyrine, intrahepatic circulatory changes could occur that depress drug clearance [23]. Alterations in metabolism or the endocrine system are more likely with the higher doses of Co-proto [22] as might also be expected for circulatory changes. In connection with the latter, electron and light microscopy have detected no organellar structural or histologic differences between control animals and those given the highest dose of Co-proto. However, the breath test data suggest that preferential isozyme depletion may account for the initial drop in aminopyrine plasma

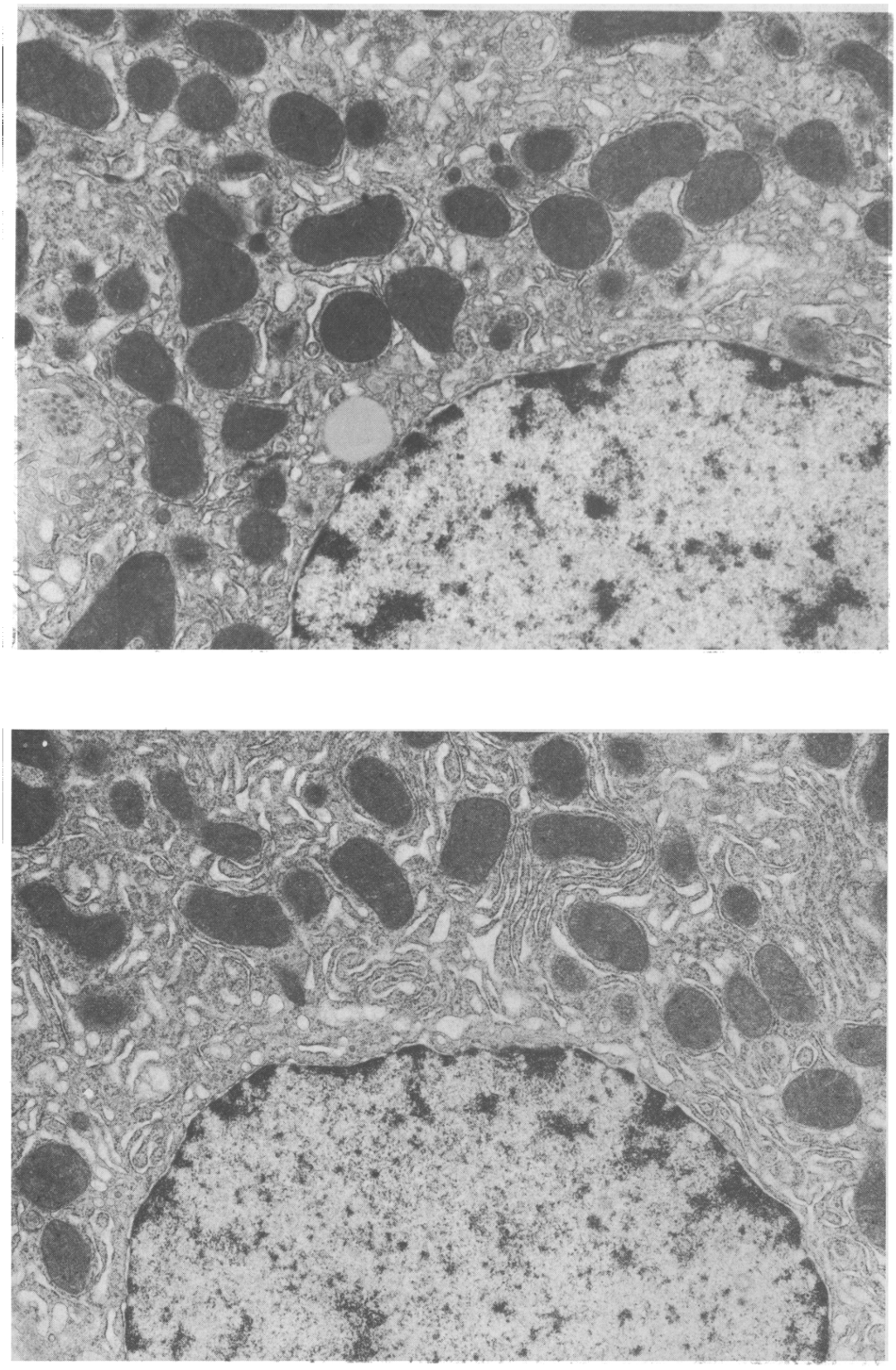

Fig. 5. Transmission electron micrographs of liver sections of control (top panel) and Co-proto-treated rats (bottom panel) at 72 hr post injection. Rats were injected with either 60 μmol Co-proto/kg rat weight or with saline. Magnification of 15,000× shows evenly dispersed chromatin, prominent smooth endoplasmic reticulum and mitochondria. No abnormalities are evident in either micrograph.

clearance since there is evidence [24, 25] that aminopyrine and caffeine are preferentially metabolized by different cytochrome P-450 isozymes. Specifically, the most straightforward interpretation of the breath test is that Co-proto treatment preferentially and potently depletes the cytochrome P-450 isozyme(s) responsible for aminopyrine modification with the first 10% of the cytochrome P-450 depletion, but depletes that involved in caffeine modification with the first 50% of depletion of the cytochrome. Involvement of apoprotein levels is implicated because heme is a positive modulator of cytochrome P-450 gene transcription [26], and Co-proto treatment decreases the amount of several cytochrome P-450 isozymes as visualized by gel electrophoresis of liver microsomes [27]. Still, other processes may also be involved, since aminopyrine undergoes two demethylations, each removing a labeled carbon, while the single labeled carbon on caffeine can be removed from either caffeine or one of its metabolites [28]. Taken together, these results imply general agreement between both *in vivo* measures of drug elimination and suggest that the model of the Co-proto-treated rat of necessity should incorporate preferential isozyme depletion. Along these lines, it is reasonable to speculate that the differential suppression of $^{14}CO_2$ evolution between aminopyrine and caffeine may also be linked to nonspecific isozyme depletion along with induction of a specific isozyme(s). Normal cytochrome P-450 turnover is relatively rapid [29, 30], and significant induction of total cytochrome P-450 [31] as well as specific isozymes [32] may occur within the 72 hr that elapse between Co-proto injection and study of these animals.

Additional evidence for preferential cytochrome P-450 isozyme depletion is provided by *in vitro* enzyme kinetics of aminopyrine demethylation. Alterations of V_{max} and K_m of a particular substrate can often be explained by shifts in the isozyme distribution [24, 25, 33–36]. The present study shows an approximately 4-fold decrease in the V_{max} and 3-fold increase in the K_m of formaldehyde formation from aminopyrine demethylation as hepatic cytochrome P-450 was depleted by Co-proto, and both changes were as expected for a decrease in cytochrome P-450-centered drug metabolism. The decrease in V_{max} was almost totally accounted for by the 70% decrease in cytochrome P-450 content, and V_{max} extrapolated approximately to zero with complete absence of cytochrome P-450. This provides a direct connection between cytochrome P-450 destruction and the *in vitro* inability to metabolize aminopyrine, and is consistent with the decrease found in aminopyrine plasma clearance. The increase in K_m suggests a shift in the isozyme distribution with Co-proto-induced cytochrome P-450 depletion since the K_m is known to be isozyme dependent [25, 37].

Even more significant to this study is the alteration in plasma clearance predicted by Co-proto-induced changes in V_{max} and K_m. For drugs of low extraction ratio (like aminopyrine) where plasma clearance is not flow limited, others have shown [38, 39] that the *in vivo* plasma clearance can be mathematically related to the V_{max} and K_m values determined by *in vitro* enzyme kinetics. Specifically, the plasma

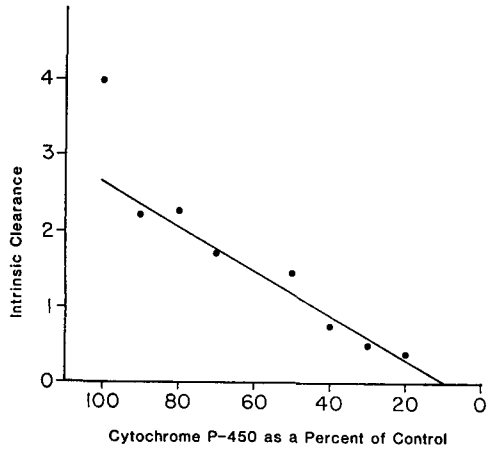

Fig. 6. Variation of intrinsic clearance with Co-proto-induced decrease in microsomal cytochrome P-450 content. Intrinsic clearance was calculated using the formula in the text with V_{max} and K_m values obtained by averaging all points within intervals of cytochrome P-450 content, e.g. 20 ± 5%, 30 ± 5%, etc., in Fig. 4, A and B.

clearance is directly proportional to the intrinsic clearance (Cl_{int}) given by

$$Cl_{int} = \frac{V_{max}}{K_m + [drug]}$$

where the drug concentration is in the liver water. In this study, the initial (maximal) aminopyrine concentration calculated from peak plasma levels was 0.25 mM, and with 30% of the drug bound to albumin [40], it can be estimated as 0.18 mM in the liver water. Figure 6 shows the variation in the intrinsic clearance (as Co-proto causes cytochrome P-450 depletion) calculated with the measured V_{max} and K_m values substituted into the equation given above. The intrinsic clearance drops by 40% with depletion of the first 10% of cytochrome P-450 and linearly thereafter. This behavior is strikingly similar to that found with aminopyrine plasma clearance in Fig. 2 and suggests that both responses to cytochrome P-450 depletion have the same underlying mechanism. Furthermore, these proportional responses have three other implications. First, alterations in the cytochrome P-450 V_{max} and K_m values are sufficient to explain the Co-proto-induced drop in hepatic clearance; neither lack of hepatic reducing equivalents nor circulatory changes are required. Second, the proportionality is consistent with the decrease in plasma clearance originating with preferential depletion of cytochrome P-450 isozymes caused by Co-proto injection. This result is the same as that suggested by the breath test. However, other factors, such as Co-proto-induced changes in phospholipid concentration and/or identity, may contribute to alteration in cytochrome P-450 enzyme kinetics without alteration of the isozyme distribution [41] (see below). Furthermore, Co-proto can incorporate into apocytochrome P-450 rendering it catalytically inactive [42], and this may not be uniformly distributed among isozymes. Third, the Co-proto-treated rat could provide further testing of mathematical models

that relate the parameters of *in vitro* enzyme kinetics to those of *in vivo* drug clearance and possibly the breath test.

Co-proto treatment decreased in a dose-dependent manner not only the cytochrome P-450 content of liver microsomes but also the cytochrome b_5 content as well as the NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase activities. This involvement of other members of the electron transport chain has several potential consequences. The ratio of NADPH–cytochrome P-450 reductase:cytochrome P-450 is about 1:15 [43], which, along with the other functions of the former, magnifies the importance of its Co-proto-induced loss. Heme oxygenase requires NADPH–cytochrome P-450 reductase for heme catabolism [44], and the decreased reductase activity may affect the partitioning of reducing equivalents between cytochrome P-450 and heme oxygenase. Also NADPH–cytochrome P-450 reductase itself can catabolize heme [45]. Furthermore, NADH–cytochrome b_5 reductase and cytochrome b_5, in addition to their function in drug metabolism, are crucial to fatty acid and steroyl desaturase activities as well as fatty acid elongation [46]. Inhibition of these activities may have secondary structural and therefore functional consequences [41, 47, 48]. Indeed, such structural changes may occur within the time frame of this study [49] and could increase with longer investigations employing the Co-proto-treated animals. Taken together, these complex interrelationships emphasize the importance of changes in all members of the electron transport chain and of potential changes in the phospholipids induced by Co-proto treatment.

This study of the Co-proto-treated rat supports its use as a model of cytochrome P-450-centered hepatic dysfunction and thus warrants a critical discussion of its application. The Co-proto-induced changes in the aminopyrine breath test appear to parallel those in plasma clearance, but the high baseline $^{14}CO_2$ evolution with both aminopyrine and caffeine makes this less clear. Indeed, cumulative $^{14}CO_2$ exhaled did not clearly extrapolate toward zero with decreasing cytochrome P-450 content as was found with other measures of hepatic metabolism. The breath test depends importantly not only on the mixed-function oxidase system but also on oxidation of multiple CO_2 precursors which are in equilibrium with multiple metabolic pools [18, 28]. Furthermore, there is evidence for extrahepatic drug demethylation [50]. Thus, there may be kinetic as well as extrahepatic contributions to the high baseline. Still, many reports [24, 50] suggest that the breath test may be a good measure of plasma clearance, and thus the breath test may be a marker of drug metabolism in the Co-proto-treated rat. Another potential weakness, especially if the Co-proto-treated animal is to be used in mathematical models mentioned previously, is that the linear decline in the intrinsic clearance extrapolates to zero at about 10% cytochrome P-450 remaining, whereas the plasma clearance extrapolates approximately to zero at total absence of this enzyme. However, considering the approximations and averaging used to construct Fig. 6, this divergence is not unexpected. Finally, it is difficult to prove unequivocally that the alterations in drug metabolism are due exclusively to changes in the distribution of cytochrome P-450 isozymes because electrons enter the *in vitro* aminopyrine demethylase assay at the level of NADPH–cytochrome P-450 reductase, which itself is decreased by Co-proto. Quantification of the cytochrome P-450 isozyme distribution after Co-proto treatment would help to clarify this problem. In any event, the predominance of a particular isozyme might be desired in certain applications of the Co-proto-treated rat model, and pentobarbital or 3-methylcholanthrene induction in combination with or prior to Co-proto-induced cytochrome P-450 depletion may prove useful in defining/adjusting the isozyme distribution. In summary, the Co-proto-treated rat should provide enhanced understanding of cytochrome P-450-centered hepatic dysfunction. However, the model should be used with the understanding that Co-proto apparently alters the cytochrome P-450 isozyme distribution as it depletes cytochrome P-450, and experimental results should be interpreted within a broad framework of potential contributions from sources other than changes in this enzyme alone.

REFERENCES

1. Maines MD and Kappas A, Cobalt induction of hepatic heme oxygenase; with evidence that cytochrome P-450 is not essential for this enzyme activity. *Proc Natl Acad Sci USA* **71**: 4293–4297, 1974.
2. Maines MD and Kappas A, Cobalt stimulation of heme degradation in the liver. *J Biol Chem* **250**: 4171–4177, 1975.
3. Chetty KN, Drummond L, Subba Rao DSV and Desaiah D, Inhibition of rat hepatic microsomal cytochrome P-450 system by cobaltous chloride and reversal of inhibition by iron *in vivo*. *Drug Chem Toxicol* **2**: 375–381, 1979.
4. Maines MD and Kappas A, Studies on the mechanism of induction of heme oxygenase by cobalt and other metals. *Biochem J* **154**: 125–131, 1976.
5. DeMatteis F and Unseld A, Increased liver haem degradation caused by foreign chemicals: a comparison of the effects of 2-alkyl-2-isopropylacetamide and cobaltous chloride. *Biochem Soc Trans* **4**: 205–209, 1976.
6. Drummond GS and Kappas A, The cytochrome P-450-depleted animal: an experimental model for *in vivo* studies in biological chemistry. *Proc Natl Acad Sci USA* **79**: 2384–2388, 1982.
7. Kappas A and Drummond GS, Control of heme metabolism with synthetic metalloporphyrins. *J Clin Invest* **77**: 335–339, 1986.
8. Yoshida T and Kikuchi G, Reaction of the microsomal heme oxygenase with cobaltic protoporphyrin IX, an extremely poor substrate. *J Biol Chem* **253**: 8479–8482, 1978.
9. Hanew T, Schenker S, Meredith CG and Henderson GT, The pharmacokinetic interaction of diethyl ether with aminopyrine in the rat. *Proc Soc Exp Biol Med* **175**: 64–69, 1984.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Estabrook RW and Werringloer J, The measurement of difference spectra: application to the cytochromes of microsomes. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 212–220. Academic Press, New York, 1978.
12. Litterst CL, Mimnaugh EG, Regan RL and Gram TE,

Comparison of *in vitro* drug metabolism by lung, liver and kidney of several common laboratory species. *Drug Metab Dispos* **3**: 259–265, 1975.
13. Nash T, The colorimetric estimation of formaldehyde by means of the Hantzch reaction. *Biochem J* **55**: 416–421, 1953.
14. Duggleby RG, A nonlinear regression program for small computers. *Analyt Biochem* **110**: 9–18, 1980.
15. Williams CH and Kamin H, Microsomal triphosphopyridine nucleotide–cytochrome *c* reductase of liver. *J Biol Chem* **237**: 587–595, 1962.
16. Kirkun G and Cederbaum AI, Evaluation of microsomal pathways of oxidation of alcohols and hydroxyl radical scavenging agents with carbon monoxide and cobalt protoporphyrin IX. *Biochem Pharmacol* **34**: 2929–2935, 1985.
17. Cheeseman KH, Albano EF, Tomasi A and Slater TF, The effect of the administration of cobaltic protoporphyrin IX on drug metabolism, carbon tetrachloride activation and lipid peroxidation in rat liver microsomes. *Chem Biol Interact* **50**: 143–151, 1984.
18. Irving CS, Schoeller DA, Nakamura K-I, Baker AL and Klein PD, The aminopyrine breath test as a measure of liver function. *J Lab Clin Med* **100**: 356–373, 1982.
19. Reinke LA, Kauffman FC, Belinsky SA and Thurman RG, Interactions between ethanol metabolism and mixed-function oxidation in perfused rat liver: inhibition of *p*-nitroanisole O-demethylation. *J Pharmacol Exp Ther* **213**: 70–78, 1980.
20. Muhoberac BB, Roberts RK, Hoyumpa AM and Schenker S, Mechanism(s) of ethanol–drug interactions. *Alcoholism: Clin Exp Res* **8**: 583–593, 1984.
21. Smith TJ, Drummond GS and Kappas A, Cobalt-protoporphyrin suppresses thyroid and testicular hormone concentrations in rat serum: a novel action of this synthetic heme analogue. *Pharmacology* **34**: 9–16, 1987.
22. Galbraith RA, Drummond GS, Krey L and Kappas A, Relationship of suppression of the androgenic axis by cobalt-protoporphyrin to its effects on weight loss and hepatic heme oxygenase induction. *Pharamcology* **34**: 241–249, 1987.
23. Nies AS, Shand DG and Branch RA, Hemodynamic drug interactions: the effects of altering hepatic blood flow on drug disposition. In: *Drug Interactions* (Eds. Morselli PL, Giarattini S and Cohen SN), pp. 231–240. Raven Press, New York, 1974.
24. Willson RA and Hart FE, The comparison of *in vivo* plasma radioactivity clearance and $^{14}CO_2$ breath elimination of model drugs in the rat: a study in regional hepatocyte function. *Toxicol Appl Pharmacol* **61**: 177–184, 1981.
25. Mannering GJ, Hepatic cytochrome P-450-linked drug-metabolizing systems. In: *Concepts in Drug Metabolism* (Eds. Jenner P and Testa B), pp. 53–166, Marcel Dekker, New York, 1981.
26. Dwarki VJ, Francis VNK, Bhat GJ and Padmanaban G, Regulation of cytochrome P-450 messenger RNA and apoprotein levels by heme. *J Biol Chem* **262**: 16958–16962, 1987.
27. Kappas A and Drummond GS, Control of heme and cytochrome P-450 metabolism by inorganic metals, organometals and synthetic metalloporphyrins. *Environ Health Perspect* **57**: 301–306, 1984.
28. Bircher J and Preisig R, Exhalation of isotopic CO_2. In: *Methods in Enzymology* (Ed. Jakoby WB), Vol. 77, pp. 3–9. Academic Press, New York, 1981.
29. Gasser R, Hauri HP and Meyer UA, The turnover of cytochrome P-450b. *FEBS Lett* **147**: 239–242, 1982.
30. Parkinson A, Thomas PE, Ryan DE and Levin W, The *in vivo* turnover of rat liver microsomal epoxide hydrolase and both the apoprotein and heme moieties of specific cytochrome P-450 isozymes. *Arch Biochem Biophys* **225**: 216–236, 1983.
31. Parkinson A, Thomas PE, Ryan DE, Reik LM, Safe SH, Robertson LW and Levin W, Differential time course of induction of rat liver microsomal cytochrome P-450 isozymes and epoxide hydrolase by aroclor 1254. *Arch Biochem Biophys* **225**: 203–215, 1983.
32. Harada N and Omura T, Phenobarbitol- and 3-methylcholanthrene-induced synthesis of two different molecular species of microsomal cytochrome P-450 in rat liver. *J Biochem (Tokyo)* **93**: 1361–1373, 1983.
33. Guengerich FP, Dannan STW, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
34. Coon MJ, Drug metabolism by cytochrome P-450: progress and perspectives. *Drug Metab Dispos* **9**: 1–4, 1981.
35. Morgan ET, Koop DR and Coon MJ, Catalytic activity of cytochrome P-450 isozyme 3a isolated from liver microsomes of ethanol-treated rabbits. *J Biol Chem* **257**: 13951–13957, 1982.
36. Åström A and DePierre JW, Rat liver microsomal cytochrome P-450: purification, characterization, multiplicity and induction. *Biochim Biophys Acta* **853**: 1–27, 1986.
37. Kaul KL and Novak RF, Induction of rabbit hepatic microsomal cytochrome P-450 by imidazole: enhanced metabolic activity and altered substrate specificity. *Arch Biochem Biophys* **235**: 470–481, 1984.
38. Rane A, Wilkinson GR and Shand DG, Prediction of hepatic extraction ratio from *in vitro* measurement of intrinsic clearance. *J Pharmacol Exp Ther* **200**: 420–424, 1977.
39. Pang KS, Rowland M and Tozer TN, *In vivo* evaluation of Michaelis–Menten constants of hepatic drug-eliminating systems. *Drug Metab Dispos* **6**: 197–200, 1978.
40. Moffat AC, Jackson JV, Moss MS, Widdop B and Greenfield ES, *Clark's Isolation and Identification of Drugs*, p. 338. Pharmaceutical Press, London, 1986.
41. Strobel HW, Lu AYH, Heideman J and Coon MJ, Phosphatidylcholine requirement in the enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon and drug hydroxylation. *J Biol Chem* **245**: 4851–4854, 1970.
42. Bonkovsky HL, Sinclair JF, Healey JF, Sinclair PR and Smith EL, Formation of cytochrome P-450 containing heme or cobalt-protoporphyrin in liver homogenates of rats treated with phenobarbitol and allylisopropylacetamide. *Biochem J* **222**: 453–462, 1984.
43. Gander JE and Mannering GJ, Kinetics of hepatic cytochrome P-450-dependent monooxygenase systems. In: *International Encyclopedia of Pharmacology and Therapeutics* (Eds. Schenkman JB and Kupfer D), Section 108, pp. 667–697. Pergamon Press, Oxford, 1982.
44. Yoshinaga T, Sassa S and Kappas A, The occurrence of molecular interactions among NADPH–cytochrome *c* reductase, heme oxygenase, and biliverdin reductase in heme degradation. *J Biol Chem* **257**: 7786–7793, 1982.
45. Yoshinaga T, Sassa S and Kappas A, A comparative study of heme degradation by NADPH-cytochrome *c* reductase alone and by the complete heme oxygenase system. *J Biol Chem* **257**: 7794–7802, 1982.
46. Oshino N, Cytochrome b_5 and its physiological significance. In: *International Encyclopedia of Pharmacology and Therapeutics* (Eds. Schenkman JB and Kupfer D) Section 108, pp. 407–447. Pergamon Press, Oxford, 1982.
47. Lu AYH and West SB, Reconstituted mammalian

mixed-function oxidases: requirements, specificities and other properties. *Pharmacol Ther (Part A)* **2**: 337–358, 1978.

48. Zakim D and Vessey DA, Regulation of microsomal enzymes by phospholipids: reproduction of uniquely modified forms of microsomal UDP-glucuronyltransferase by treatment with phospholipase A and detergents. *Biochim Biophys Acta* **410**: 61–73, 1975.
49. Houslay MD and Stanley KK, *Dynamics of Biological Membranes*, p. 254. John Wiley, Chichester, 1982.
50. Lauterburg BH and Bircher J, Expiration measurement of maximal aminopyrine demethylation *in vivo*: effect of phenobarbital, partial hepatectomy, portacaval shunt and bile duct ligation in that rat. *J Pharmacol Exp Ther* **196**: 501–509, 1976.