0026-895X/87/030423-09$02.00/0

MOLECULAR PHARMACOLOGY, 32:423-431

Contribution of Aniline Metabolites to Aniline-Induced Methemoglobinemia

JAMES H. HARRISON, JR.,[1] and DAVID J. JOLLOW

Department of Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425

Received June 10, 1986; Accepted June 12, 1987

SUMMARY

Methemoglobinemia after aniline and certain aniline derivatives is thought to be mediated by toxic metabolites formed during the hepatic clearance of the parent compounds. However, three aniline metabolites—phenylhydroxylamine, 2-aminophenol, and 4-aminophenol—catalyze methemoglobin formation in erythrocyte suspensions and, hence, could contribute to methemoglobin formation *in vivo* after aniline. To determine the relative contributions of these aniline metabolites to aniline-induced methemoglobinemia in rats, we determined time courses of methemoglobinemia in rat erythrocyte suspensions and in rats after treatment with 2- and 4-aminophenol, phenylhydroxylamine, and aniline. The relative potencies for methemoglobin production *in vitro* after phenylhydroxylamine, 2-aminophenol, and 4-aminophenol were about 10:5:1, based on both peak and area of the methemoglobin versus time curve. Approximate minimum concentrations for observable methemoglobin formation *in vitro* from these compounds were 20, 50, and 200 μM, respectively. Compared with the *in vitro* data, the relative potencies of the aminophenols for methemoglobinemia in rats after intraperitoneal injections were reduced with respect to phenylhydroxylamine (to 100:4:1, respectively), apparently as a result of rapid *in vivo* clearance of the aminophenols. Subsequent experiments, in which the time courses of the aniline metabolites were determined in blood after toxic doses of aniline, demonstrated that only phenylhydroxylamine (measured as phenylhydroxylamine + nitrosobenzene) accumulated to blood levels exceeding the minimum concentration required for methemoglobin production *in vitro*. In addition, blood levels of phenylhydroxylamine remained in the toxic range throughout most of the methemoglobinemic response after aniline treatment. These data are consistent with phenylhydroxylamine being the sole mediator of aniline-induced methemoglobinemia in these rats.

Methemoglobinemia is a well known toxic effect that occurs in animals and humans after administration of aniline and certain aniline derivatives (1). These compounds do not cause hemoglobin oxidation when incubated with erythrocyte suspensions at physiologically relevant concentrations (1-3). It therefore is generally accepted that the toxicity to erythrocytes results from the action of active metabolites formed *in vivo* during hepatic clearance of the parent compounds (4).

Aniline-induced methemoglobinemia in experimental animals has been studied extensively as a model for methemoglobinemia after arylamines in general. Several aniline metabolites, including phenylhydroxylamine, 2-aminophenol, and 4-aminophenol, have been considered as possible mediators of aniline-induced methemoglobinemia. All three compounds are direct-acting; that is, they cause significant formation of methemoglobin when added to erythrocyte suspensions *in vitro* (1). Phenylhydroxylamine, which is interconvertible with nitrosobenzene in blood (5), is the most potent of the metabolites for the production of methemoglobin *in vivo* (1). It is detectable in blood (6), but not urine (7) after aniline administration. Although less potent *in vivo* than phenylhydroxylamine, 2- and 4-aminophenol are present (in conjugated forms) as major urinary metabolites of aniline (8) and are considered to be produced from aniline *in vivo* in greater quantities than phenylhydroxylamine; recent studies suggest that they may mediate methemoglobinemia after aniline or aniline derivatives in humans (9).

To evaluate the relative contributions of these aniline metabolites to aniline-induced methemoglobinemia, we determined the time courses of methemoglobin, aniline, and aniline metabolites (phenylhydroxylamine, 2-aminophenol, and 4-aminophenol) in the blood of rats after intraperitoneal injections of aniline. Comparison of these time course data with potency data for the individual metabolites derived from erythrocyte suspension studies and *in vivo* experiments demonstrated which metabolites were present *in vivo* after aniline in sufficient concentrations to cause significant methemoglobinemia.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 200-250 g were obtained from Camm Research, Inc. (Wayne, NJ) and were maintained

This work was supported by Grant HL30038 from the United States Public Health Service.

[1] Present address: Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

ABBREVIATIONS: HPLC, high pressure liquid chromatography.

in hanging stainless steel cages on standard lab chow (Wayne Lab Blox, Allied Mills, Chicago, IL) and water, *ad libitum*.

Chemicals. Aniline HCl and nitrosobenzene were purified, and phenylhydroxylamine was prepared and purified, as described previously (10). Aminophenols (2-, 3-, and 4-, reagent grade, Aldrich Chemical Co., Milwaukee, WI) were purified by repetitive sublimation. The above compounds were stored in the dark under N_2 at −20°C. All other chemicals were reagent grade and were used without further purification. Chromatography solvents were "distilled-in-glass" grade (Burdick-Jackson Laboratories, Muskegan, MI). Other solvents were reagent grade (Fisher Chemical Co., Fairlawn, NJ).

Measurement of aniline and aniline metabolites in rat blood. Aniline in rat blood was separated and quantified by differential extraction (11) after intraperitoneal injections of generally labeled, tritiated aniline HCl (222 μCi/kg, 1.5 mmol/kg) in saline. 2-Aminophenol, 4-aminophenol, and 4-hydroxyacetanilide in rat plasma were assayed by reverse-phase HPLC with electrochemical detection, by a modification of the method of Sternson and Dewitte (12), as described previously (11). The combined amount of phenylhydroxylamine and nitrosobenzene in rat blood samples after intraperitoneal injections of aniline HCl was assayed by HPLC with electrochemical detection (10). Blood time courses for all these compounds were determined in individual rats after aniline treatment by assaying serial 75-μl blood samples taken from the orbital sinus under manual restraint, as described previously (11).

Erythrocyte suspensions. Rat erythrocytes were collected into a citrate-phosphate-dextrose anticoagulant (13) by cardiac puncture. The erythrocytes were washed twice in 0.9% saline and suspended at a hematocrit of 50% in phosphate-buffered saline (110 mM sodium chloride, 20 mM disodium hydrogen phosphate, and 4 mM potassium dihydrogen phosphate; pH = 7.4) with 10 mM glucose. Aniline HCl, 2-aminophenol, or 4-aminophenol was placed in scintillation vials in a small volume of methanol and blown to dryness under nitrogen. Under these conditions, more than 95% of the compounds were recovered in aqueous solution after 1 min of gentle swirling with 3 ml of water. Phenylhydroxylamine or nitrosobenzene was placed in the vials in 10 μl of acetone. We began the experiments by adding 3-ml erythrocyte suspensions to each vial. During the experiments, the vials were loosely capped and maintained in a water bath at 37°C with mild shaking. Samples (100 μl) of the suspensions were removed at intervals for assay of methemoglobin.

Methemoglobin levels relative to total hemoglobin in the suspensions were measured by a modification of the spectrophotometric technique of Evelyn and Malloy (14). Samples of the suspensions (100 μl) were hemolyzed with 5 ml of phosphate buffer (0.277% potassium dihydrogen phosphate, 0.289% disodium hydrogen phosphate, and 0.05% Triton X-100; pH 7.8). The hemolyzed solution was divided into four aliquots; one drop of aqueous 10% KCN was added to aliquots nos. 2 and 4, and one drop of aqueous 20% $KFe(CN)_6$ was added to aliquots nos. 3 and 4. After mixing, the absorbance of each aliquot at 635 nm was measured, yielding A_1, A_2, A_3, and A_4. The percentage of the total hemoglobin present as methemoglobin was then calculated:

$$\% \text{ methemoglobin} = \frac{A_1 - A_2}{A_3 - A_4} 100.$$

Experiments *in vivo*. Aniline HCl, 2-aminophenol, 4-aminophenol, and phenylhydroxylamine were injected intraperitoneally into rats in 1.0 ml of vehicle per kilogram body weight. Aniline HCl was dissolved in 0.9% saline; 2- and 4-aminophenol were dissolved in dilute HCl and were titrated to pH 6–6.5 with NaOH before injection. Phenylhydroxylamine was dissolved in 0.9% saline containing 5% Tween-80. In some experiments, galactosamine, a noncompetitive inhibitor of hepatic glucuronidation of phenols (19), was dissolved in 0.9% saline and injected intraperitoneally (200 mg/kg) 30 min before the test compounds. Control blood samples were obtained from the orbital sinus in 75-μl heparinized capillary tubes immediately before injection of the test compounds. After injection, serial orbital sinus blood samples were collected until methemoglobin levels returned to baseline values. Blood samples to be assayed for methemoglobin were hemolyzed and assayed immediately after collection, as described above. For quantification of aniline and phenolic aniline metabolites in plasma, serial orbital sinus blood samples were collected in 75-μl heparinized hematocrit capillaries after intraperitoneal injection of the test compound, centrifuged to separate plasma for erythrocytes, and immediately frozen at −20°C. Samples were assayed by HPLC with electrochemical detection as described above within 8 hr of collection. Blood samples to be assayed for phenylhydroxylamine and nitrosobenzene were extracted and analyzed immediately as described previously (10). Aniline, 4-hydroxyacetanilide, 2-aminophenol, and 4-aminophenol in plasma were stable for at least 12 hr at −20°C. Samples stored for more than 24 hr showed some loss of the aminophenols (15–20%).

Areas under the methemoglobin time course curves were estimated by fitting curves by eye to data from individual cell suspensions or animals plotted on graph paper, then counting the squares under the curves. Estimates of relative potency were made by inspection of dose-response data plotted on logit paper. Statistical analyses used the Student's *t* test for pooled samples (15).

Results

Methemoglobin formation by aniline and aniline metabolites *in vitro*. To determine the relative potencies of 2-aminophenol, 4-aminophenol, and phenylhydroxylamine for methemoglobin production *in vitro*, we incubated rat erythrocyte suspensions with aniline or aniline metabolites, and measured methemoglobin time courses. Control erythrocyte suspensions incubated at 37°C for 6 hr showed a constant methemoglobin baseline of approximately 3.5–5.0% of total hemoglobin which is consistent with an approximately normal rate of spontaneous methemoglobin production (16). Suspensions incubated with 1 mM aniline showed no increase in methemoglobin levels over these baseline values during a 2-hr time course, confirming previous studies indicating that, in physiologically relevant concentrations, aniline does not induce methemoglobin formation *in vitro* (2). In contrast, erythrocyte suspensions incubated with 2-aminophenol, 4-aminophenol, phenylhydroxylamine, or nitrosobenzene showed extensive methemoglobin formation.

In 2-aminophenol-treated incubates, the early rate of methemoglobin formation and the maximal level of methemoglobin reached during the 6-hr incubation period were both dependent on the concentration of 2-aminophenol (Fig. 1A). Peak methemoglobin levels were reached 1–2 hr after the beginning of the incubation. In erythrocytes incubated with 4-aminophenol, peak methemoglobinemia corresponding to approximately 35% of total hemoglobin was reached after 1–2 hr of incubation with 1000 μM 4-aminophenol (Fig. 1B). Increasing the 4-aminophenol concentration to 2000 μM did not further increase the peak methemoglobin concentration. The presence of a methemoglobin maximum after 4-aminophenol treatment has been noted previously *in vitro* (17) and *in vivo* (18).

In erythrocyte suspensions incubated with phenylhydroxylamine, the early rate and maximum level of methemoglobin formation were also concentration dependent (Fig. 1C). However, the early rate of methemoglobin formation was much more rapid, and maximal methemoglobin levels were reached more quickly (within 20–30 min after the start of incubation), than in aminophenol-treated suspensions. Incubation of erythrocytes with nitrosobenzene (Fig. 1D) produced methemoglobin time courses essentially identical to similar concentrations of phenylhydroxylamine, consistent with previous studies indica-

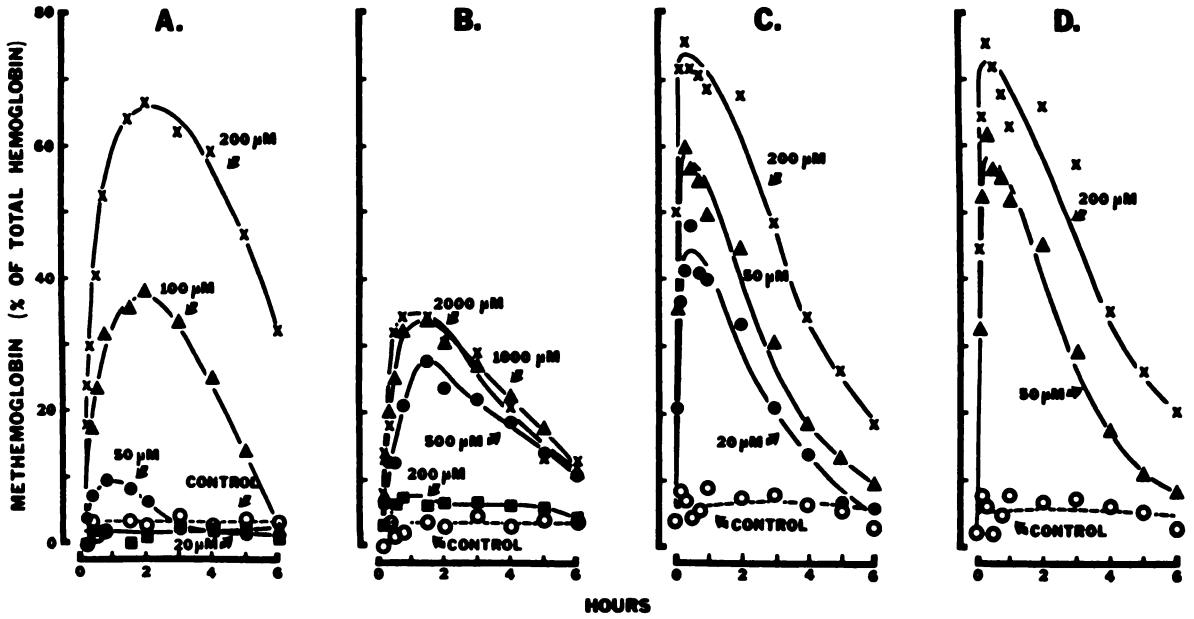

Fig. 1. Methemoglobin formation in rat erythrocytes treated with aniline metabolites. A, 2-aminophenol; B, 4-aminopenol; C, phenylhydroxylamine; and D, nitrosobenzene. Values shown are the means of five incubates.

ting that phenylhydroxylamine and nitrosobenzene are interconvertible within erythrocytes (1).

The potency relationships for peak methemoglobin and total methemoglobin (as measured by area under the methemoglobin time course curve) are summarized in Fig. 2. For each aniline metabolite, the relationship between peak and total methemoglobin was constant over the dose range tested (i.e., the curves for peak methemoglobin and total methemoglobin were parallel). Phenylhydroxylamine was the most effective methemoglobin-producing agent at low doses, producing significant levels of methemoglobin at concentrations of 20 μM. 2-Aminophenol was less effective at low concentrations, requiring levels of 50–60 μM for the production of significant amounts of methemoglobin. However, the concentration relationship of 2-aminophenol-induced methemoglobin formation was much steeper than that of phenylhydroxylamine, such that at higher concentrations, peak methemoglobin levels and total methemoglobin production after 2-aminophenol were compared to values observed after phenylhydroxylamine. 4-Aminophenol was much less potent than 2-aminophenol and phenylhydroxylamine for both peak and area under the curve, producing significant levels of methemoglobin only at concentrations greater than 200 μM. The relative potency of phenylhydroxylamine:2-aminophenol:4-aminophenol *in vitro* was approximately 10:5:1 (comparing the apparent midpoints of the dose-response curves).

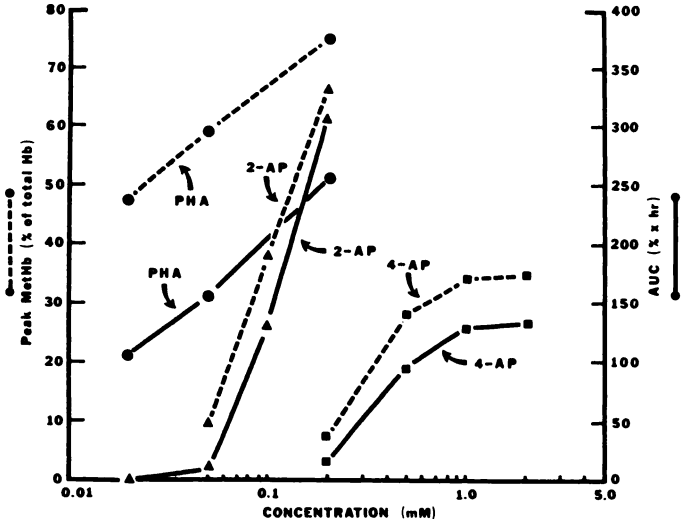

Fig. 2. Potency relationships for methemoglobin formation in rat erythrocyte suspensions incubated with aniline metabolites. *Peak MetHb*, the maximal level of methemoglobin achieved during each incubation; *AUC*, area under the methemoglobin time course curve for each incubation (1 unit area = 1% methemoglobin above baseline for 1 hr); *PHA*, phenylhydroxylamine; *2-AP*, 2-aminophenol; *4-AP*, 4-aminophenol.

Methemoglobinemia after aniline and aniline metabolites *in vivo*. The time course of methemoglobinemia was determined in rats after intraperitoneal injections of 2-aminophenol, 4-aminophenol, or phenylhydroxylamine (Fig. 3). Both peak methemoglobin levels and the duration of methemoglobinemia were dose dependent for all three aniline metabolites. Phenylhydroxylamine produced the most rapid rise in methemoglobin levels (Fig. 3C): peak values were reached within 5 min after injection. In rats treated with 2-aminophenol or 4-aminophenol, the early rates of methemoglobin formation (Fig. 3, A and B) appeared to be more rapid *in vivo* than in erythrocyte suspensions (Fig. 1, A and B), and peak methemoglobin levels occurred earlier *in vivo* (20–30 min after administration, in contrast to 1–2 hr *in vitro*). Both 2-aminophenol and phenylhydroxylamine were highly effective, producing levels of methemoglobinemia reaching 70–80% of total hemoglobin. 4-Aminophenol was much less effective, producing maximal methemoglobin levels of less than 30% even after 2 mmol/kg.

Time courses of methemoglobinemia in rats after intraperitoneal doses of aniline are shown in Fig. 4. Maximal methemoglobin levels were reached 1–2 hr after administration of the dose, with the time-to-peak appearing to increase with dose. Although the dose-dependent increase in peak methemoglobin levels is significant, reaching a maximum of approximately 45% of total hemoglobin at the highest dose level tested, the most striking effect of increasing aniline dose is prolongation of the

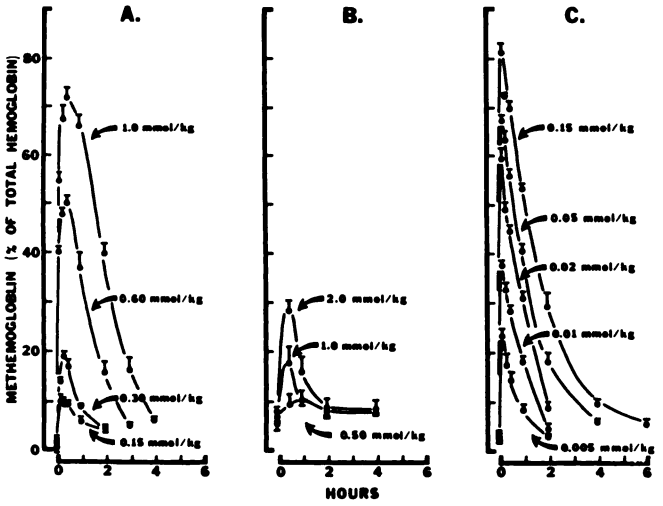

Fig. 3. Methemoglobinemia in rats after treatment with aniline metabolites. Rats received the indicated doses of 2-aminophenol (A), 4-aminophenol (B), or phenylhydroxylamine (C) by intraperitoneal injection. Initial (O = T) blood samples were taken from the orbital sinus immediately before injection of the test compounds. Serial orbital sinus blood samples then were collected from each animal and assayed immediately for methemoglobin. *Error bars* indicate standard deviations (n = 5).

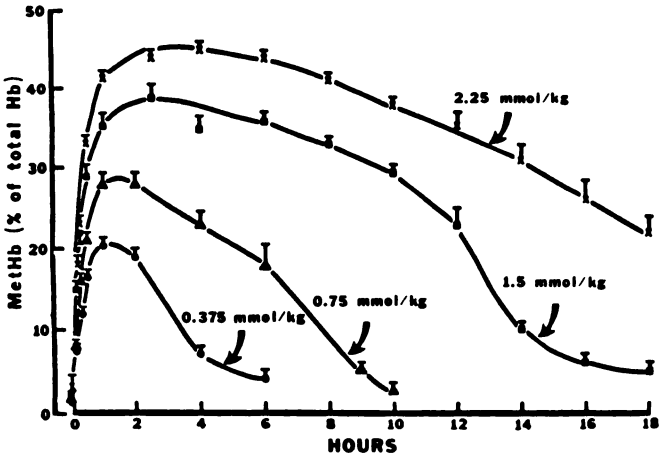

Fig. 4. The effect of aniline dose on aniline-induced methemoglobinemia in rats. Rats received the indicated doses of aniline by intraperitoneal injection. Initial (O = T) blood samples were taken from the orbital sinus immediately before injection of aniline. Serial blood samples then were collected from the orbital sinus of each animal and assayed immediately for methemoglobin. *Error bars* indicate standard deviations (n = 5).

duration of methemoglobinemia. Whereas methemoglobinemia is essentially terminated by 6 hr after a 0.375 mmol/kg dose, more than 20% methemoglobinemia remains 18 hr after a 2.25 mmol/kg dose.

The relative potencies of 2-aminophenol, 4-aminophenol, phenylhydroxylamine, and aniline HCl for peak and total methemoglobinemia (area under the methemoglobinemia time course curve) after intraperitoneal injection are shown in Fig. 5. Phenylhydroxylamine was the most potent of these compounds for the production of methemoglobin *in vivo*. The comparatively shallow slope of the phenylhydroxylamine potency curve noted in the cell suspensions (Fig. 2) was also seen *in vivo*. In contrast to the potency ratios determined *in vitro*, the *in vivo* potency ratio for phenylhydroxylamine:2-aminophenol:4-aminophenol was approximately 100:4:1. Aniline was intermediate in potency between 2-aminophenol and 4-aminophenol for peak methemoglobinemia. For total methemoglobin production, the potency of aniline was between that of 2- and 4-aminophenol, but aniline caused the formation of a much larger total amount of methemoglobin at higher doses due to the dramatic increase in the duration of methemoglobinemia seen at these doses of aniline (see Fig. 4).

Clearance of 2-aminophenol in erythrocyte suspensions and *in vivo*. To determine whether the dramatic increase in the phenylhydroxylamine:aminophenols potency ratios *in vivo* (100:4:1), compared to *in vitro* (10:5:1), might result from rapid clearance of the aminophenols *in vivo* (by, for example, conjugation reactions involving the phenolic group), the elimination rate of 2-aminophenol was determined in erythrocyte suspensions and in rats. In erythrocyte suspensions, 2-aminophenol concentration declined in a first order manner from a starting value of 10 μg/ml, with a half-life of approximately 82 min (data not shown). The elimination of 2-aminophenol *in vivo* after intraperitoneal injection (0.50 mmol/kg) was also first order, but the rate of removal was much more rapid than in cell suspensions, with a half-life of 4.4 ± 0.17 min (Fig. 6). In fats pretreated with galactosamine, the half-life of 2-aminophenol was approximately doubled (Fig. 6), and the peak and area under the curve of methemoglobinemia after 2-aminophenol were increased (Table 1). Galactosamine is known to inhibit, noncompetitively, the hepatic glucuronidation of phenols (19). The peak and area of methemoglobinemia after phenylhydroxylamine (which is thought not to be cleared by glucuronidation), were not significantly changed by galactosamine pretreatment (Table 1). These findings strongly suggest that the decreased potency of 2-aminophenol with respect to phenylhydroxylamine *in vivo* results from a more rapid clearance of 2-aminophenol *in vivo*.

Blood time courses of aniline metabolites *in vivo*. To determine which aniline metabolites are present *in vivo* after aniline administration in concentrations required for significant methemoglobin formation, the time courses of unconjugated 2-aminophenol, 4-aminophenol, 4-hydroxyacetanilide, and phenylhydroxylamine were measured in the blood of rats after aniline treatment (1.5 mmol/kg, ip, Fig. 7). Phenylhydroxylamine in blood was determined by measurement of the sum of phenylhydroxylamine + nitrosobenzene (10). Of these compounds, phenylhydroxylamine + nitrosobenzene were the predominant unconjugated aniline metabolites present during the first 5 hr after aniline administration. Peak levels of phenylhydroxylamine + nitrosobenzene were achieved rapidly, occurring within 10 min after aniline treatment. 4-Aminophenol concentration showed low levels initially, with a late rise, reaching a peak level of about 5 nmol/ml between 6 and 8 hr after aniline administration. 4-Hydroxyacetanilide showed a similar late rise in blood levels, reaching maximal levels of about 30 nmol/ml 6–8 hr after aniline treatment.

Free 2-aminophenol could not be detected in rat blood after intraperitoneal injection of aniline, consistent with the rapid elimination of 2-aminophenol *in vivo* (see Fig. 6). The sensitivity limit of the HPLC assay for 2-aminophenol was 0.5 nmol/ml, and hence free 2-aminophenol levels in the experiment must always have been less than this value.

Effect of galactosamine on aniline-induced methemoglobinemia. In order to confirm that the concentration of 2-aminophenol in blood is too low to contribute significantly to methemoglobinemia occurring after aniline, the time course of

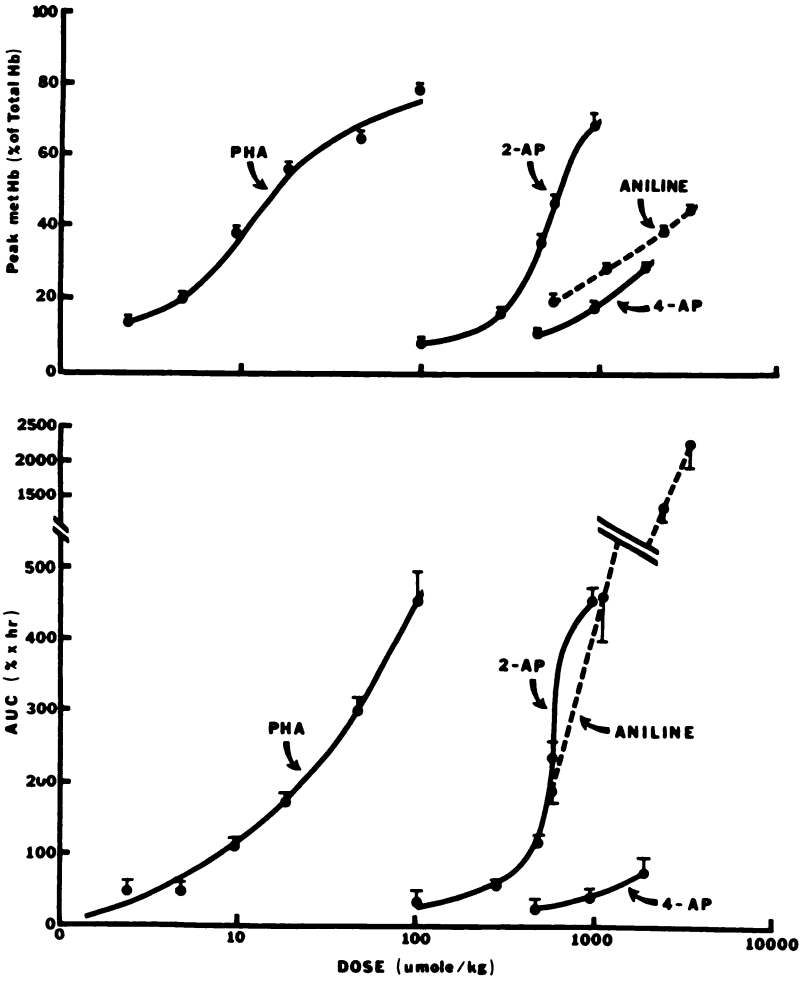

Fig. 5. Dose-response relationships for methemoglobinemia after aniline and aniline metabolites in rats. *Peak metHb*, the maximal level of methemoglobinemia observed after each dose; *AUC*, area under the methemoglobinemia time course (1 unit area = 1% methemoglobinemia above baseline for 1/3 hr); *PHA*, phenylhydroxylamine; *2-AP*, 2-aminophenol; *4-AP*, 4-aminophenol. *Error bars* indicate standard deviations (n = 5).

aniline-induced methemoglobinemia was determined in rats with and without pretreatment with galactosamine. Galactosamine inhibits 2-aminophenol clearance (Fig. 6) and potentiates 2-aminophenol-induced methemoglobinemia *in vivo*, but not that of phenylhydroxylamine (Table 1). Galactosamine had no effect on aniline clearance (data not shown), indicating that the major oxidative pathways of aniline elimination were not inhibited by galactosamine. Thus, if 2-aminophenol contributes significantly to methemoglobin production *in vivo* after aniline, galactosamine pretreatment should enhance aniline-induced methemoglobinemia. Experimentally, galactosamine pretreatment did not enhance aniline-induced methemoglobinemia (Table 1).

Clearance of aniline *in vivo*. Aniline elimination after low doses (0.15 and 0.375 mmol/kg, intraperitoneally) approximated first order kinetics with a half-life of approximately 16 min at the 0.15 mmol/kg dose level (Fig. 8). As the dose was increased (0.75–2.25 mmol/kg, intraperitoneally), the kinetics of elimination of aniline became complex. The plasma time course of aniline at the higher doses was marked by a capacity-limited phase of clearance occurring between 1 and 6 hr after aniline administration. This phase differed from zero order capacity-limited elimination, in that it appeared to be linear on a semilog plot. Subsequently, the apparent rate constant for aniline elimination increased, and a curvilinear terminal phase of elimination occurred in which the apparent rate constant approached the elimination rate constant for low doses of aniline.

Discussion

Aniline induced a dose-dependent methemoglobinemia in rats after intraperitoneal injection, but did not cause methemoglobin formation when it was present in erythrocyte suspensions in concentrations associated with *in vivo* methemoglobinemia. This finding is consistent with previous reports and with the conclusion that methemoglobinemia after aniline is mediated by one or more toxic metabolites formed during the hepatic clearance of aniline (5). The known pathways of aniline clearance are shown in Fig. 9. Parke (20) and Kao *et al.* (8) demonstrated that 2-aminophenol, 4-aminophenol, and 4-hydroxyacetanilide (as their sulfate and glucuronide conjugates) are major urinary metabolites of aniline. Although phenylhydroxylamine, nitrosobenzene, or further breakdown products of this metabolic pathway have not been demonstrated in urine after aniline (7), Kiese (6) has previously shown that nitrosobenzene is present in blood after aniline administration. Production of nitrosobenzene and/or phenylhydroxylamine from aniline has also been observed in microsomal systems (21) and in the isolated perfused rat liver (22).

Previous studies have shown that acetanilide is much less potent than aniline for methemoglobin formation *in vivo* (2), does not produce methemoglobin *in vitro* (2), and requires deacetylation for activity *in vivo* (23). Thus, attention has been focused on 2-aminophenol, 4-aminophenol, and phenylhydroxylamine as possible mediators of aniline-induced methemoglobinemia. Each of these compounds is an effective methemoglo-

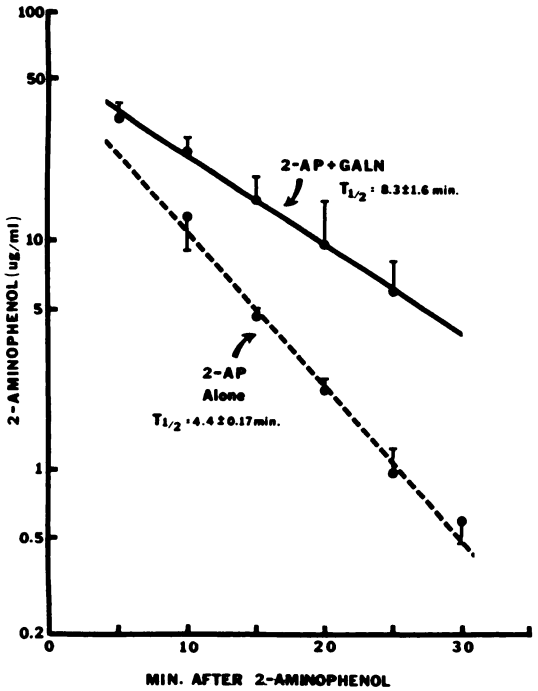

Fig. 6. The effect of galactosamine pretreatment on the plasma half-life of 2-aminophenol in rats. 2-Aminophenol (0.50 mmol/kg in saline) was injected intraperitoneally (IP). Galactosamine (200 mg/kg in saline, IP) was administered 30 min before 2-aminophenol. Serial blood samples were collected from the orbital sinus of each animal and assayed for 2-aminophenol by HPLC with electrochemical detection. *2-AP*, 2-aminophenol; *GALN*, galactosamine. *Error bars* indicate standard deviations and $T_{\frac{1}{2}}$ values are expressed as means ± standard deviations (n = 4).

bin-producing agent in erythrocyte suspensions. Since all three of the compounds are also reducing agents, their capacity to oxidize heme has been of interest. The aminophenols are thought to be converted within erythrocytes to quinonimines which then oxidize hemoglobin, whereas phenylhydroxylamine is thought to participate in a coupled oxidation with oxyhemoglobin, forming nitrosobenzene, methemoglobin, and partially reduced oxygen species. The quinonimes and phenylhydroxylamine may subsequently be regenerated within erythrocytes, completing cyclic mechanisms which may lead to the production of many equivalents of methemoglobin for each equivalent of "oxidizing" agent (1). Theoretically, the relative contribution of each of these metabolites to aniline-induced methemoglobinemia may be expected to be determined by its intrinsic potency for methemoglobin production and by the level of exposure of erythrocytes to the compound. In turn, *in vivo* blood levels of each metabolite would be determined by its kinetics of formation and elimination.

In erythrocyte suspensions, phenylhydroxylamine was the most potent of the metabolites tested for methemoglobin production (Fig. 2). 2-Aminophenol was about half as potent as phenylhydroxylamine, and 4-aminophenol was the least potent of the aniline metabolites. The potency ratio *in vitro* appeared to be about 10:5:1 for phenylhydroxylamine, 2-aminophenol, and 4-aminophenol, respectively.

In the *in vivo* studies, although a similar rank order of potency for the aniline metabolites was seen, the relative potencies of the aminophenols with respect to phenylhydroxylamine were very much decreased. The corresponding *in vivo* potency ratio was about 100:4:1. These data suggest that extraerythrocytic factors were operating *in vivo* to reduce the effectiveness of the aminophenols. Experiments to determine the elimination kinetics of 2-aminophenol demonstrated that the *in vivo* half-life of 2-aminophenol (Fig. 6) was approximately 20-fold shorter that its *in vitro* half-life. Thus, rapid elimination of 2-aminophenol *in vivo* appeared to decrease the methemoglobinemic response to this compound. This formulation is consistent with the finding of a more rapid onset and earlier peak of methemoglobinemia after 2-aminophenol treatment *in vivo* than *in vitro* (Fig. 3A versus Fig. 1A): The initial rapid accumulation of methemoglobin occurring after intraperitoneal injection of 2-aminophenol (Fig. 3A) was consistent with the high blood levels of 2-aminophenol seen 10 min after injection (ca. 200 μM, Fig. 6). Similar concentrations of 2-aminophenol *in vitro* produced comparable methemoglobin levels within 10–15 min (Fig. 1A). Subsequent rapid elimination of 2-aminophenol appeared to terminate hemoglobin oxidation, producing an early and attenuated peak methemoglobinemia *in vivo*. A similar shift in the time-to-peak methemoglobin also appeared to be occurring in the case of 4-aminophenol. Further experiments demonstrated that the elimination of 2-aminophenol is inhibited (Fig. 6) and methemoglobinemia after 2-aminophenol is enhanced in animals pretreated with galactosamine (Table 1), suggesting that rapid hepatic clearance of 2-aminophenol is an important factor contributing to the difference between the *in vitro* and *in vivo* potency ratios of phenylhydroxylamine and 2-aminophenol.

It is of interest that the time course of methemoglobinemina in rats given aniline is significantly different from that seen after treatment of the rats with the various metabolites of aniline. Aniline induced a much longer duration of methemo-

TABLE 1

Effect of galactosamine on methemoglobinemia after 2-aminophenol, phenylhydroxylamine, and aniline

Treatment	Methemoglobinemia[a]			
	Peak (%)[b]		AUC (units)[c]	
	Control	Galactosamine[d]	Control	Galactosamine[d]
2-Aminophenol (0.50 mmol/kg, IP[e])	33.2 ± 5.4	47.2 ± 10.4[f]	144.7 ± 40.1	213.4 ± 56.3[f]
Phenylhydroxylamine (0.01 mmol/kg, IP)	37.3 ± 9.1	38.8 ± 8.5	69.4 ± 14.0	85.4 ± 20.0
Aniline (1.5 mmol/kg, IP)	47.4 ± 5.3	50.9 ± 2.6	1128 ± 162	1176 ± 39

[a] Methemoglobinemia was followed for 2 hr (phenylhydroxylamine), 3 hr (2-aminophenol), or 12 hr (aniline). Values are means ± SD (n = 4 or 5).
[b] Methemoglobin is expressed as a percentage of total hemoglobin in whole blood.
[c] AUC, area under the methemoglobinemia time course curve (1 unit = 1% methemoglobinemia for ⅓ hr).
[d] Galactosamine (200 mg/kg) was injected IP 30 min prior to the test compounds.
[e] IP, intraperitoneal.
[f] $p < 0.05$.

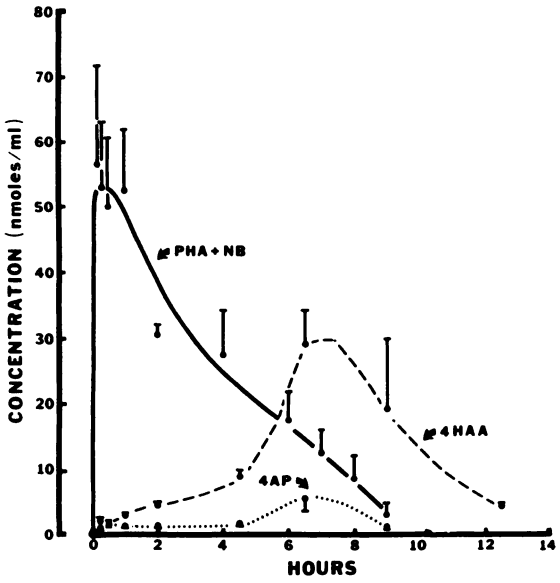

Fig. 7. Blood time courses of aniline metabolites in rats after aniline treatment. Serial orbital sinus blood samples were collected from each animal following intraperitoneal aniline administration (1.5 mmol/kg in saline). The combined concentration of phenylhydroxylamine and nitrosobenzene (*PHA + NB*) in blood was assayed by HPLC with detection by electrochemical reduction. 4-Aminophenol (*4AP*) and 4-hydroxyacetanilide (*4HAA*) were assayed in blood by HPLC with detection by electrochemical oxidation. *Error bars* indicate standard deviations (n = 4).

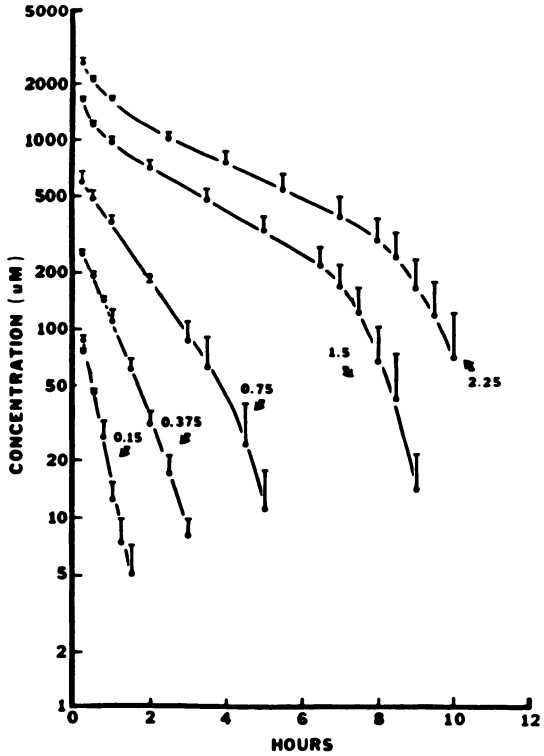

Fig. 8. The effect of aniline dose on the elimination of aniline from the blood of rats. Tritiated aniline HCl in the indicated doses (mmol/kg) was administered intraperitoneally in saline at T = O. Serial orbital sinus blood samples were collected from each rat and assayed for aniline by differential extraction. *Error bars* indicate standard deviations (n = 4).

globinemia for a given methemoglobin peak level than did any of its metabolites. Whereas the metabolites produced parallel increases in peak and total methemoglobinemia with increasing dose (Figs. 3 and 5), higher doses of aniline were associated with a disproportionate increase in the duration (and thus the AUC) of methemoglobinemia (Figs. 4 and 5). *In vivo* aniline clearance studies (Fig. 8) demonstrated that the dose-related increase in methemoglobinemia duration is accompanied by capacity limitation of aniline elimination. It seems likely that the resulting increased availability of plasma aniline after larger doses may be associated with sustained production of a toxic aniline metabolite and a resulting sustained methemoglobinemia. In this respect, it is interesting to note that our previously described elimination pattern of phenylhydroxylamine + nitrosobenzene after a 1.5 mmol/kg dose of aniline (10) closely matches the elimination pattern of aniline at that dose (Fig. 8). The half-life of the linear phase of phenylhydroxylamine + nitrosobenzene clearance under these conditions (3.5 hr) is comparable to the "half-life" of the capacity-limited phase of aniline clearance, and the onset of the terminal rapid elimination phase begins at the same time after aniline administration in each case. These findings suggest that the shape of the elimination curve of phenylhydroxylamine + nitrosobenzene is determined by the shape of the elimination curve of the parent compound, aniline.

Determination of the blood concentrations of free 2-aminophenol, 4-aminophenol, and phenylhydroxylamine + nitrosobenzene after toxic doses of aniline revealed that phenylhydroxylamine + nitrosobenzene were the only aniline metabolites that reached blood concentrations above the minimum required for significant methemoglobin formation in erythrocyte suspensions. 2-Aminophenol concentrations after aniline were always below 0.5 μM, whereas concentrations of 50–60 μM were required to produce significant methemoglobin in erythrocyte suspensions (Fig. 2). Galactosamine, which blocks 2-aminophenol clearance and potentiates 2-aminophenol-induced methemoglobinemia, did not affect aniline-induced methemoglobinemia (Table 1). The maximal 4-aminophenol concentration after aniline was approximately 5 μM, well below the 200 μM concentration required for significant methemoglobin formation in cell suspensions (Fig. 2). Further, phenylhydroxylamine + nitrosobenzene reached blood levels after aniline consistent with the degree of methemoglobinemia produced by aniline, and remained present in toxic levels over most of the time course of aniline-induced methemoglobinemia (Fig. 7). It is of interest that the elimination of phenylhydroxylamine + nitrosobenzene after aniline treatment (Fig. 7) is more rapid than methemoglobin reduction after aniline (Fig. 4). This finding is consistent with the relatively shallow concentration- and dose-response relationships seen for phenylhydroxylamine-induced methemoglobin production *in vitro* and *in vivo* (Figs. 2 and 5). It is also possible that phenylhydroxylamine and/or nitrosobenzene induce erythrocytic damage over a long period of exposure *in vivo*, affecting the capacity of the cells to reduce methemoglobin.

These experiments indicate that it is very unlikely that 2- or 4-aminophenol contributed at all to aniline-induced methemoglobinemia in these rats. Phenylhydroxylamine appears to be the sole toxic metabolite mediating aniline-induced methemoglobinemia. Although the possibility that other, unknown aniline metabolites contribute has not been ruled out, phenyl-

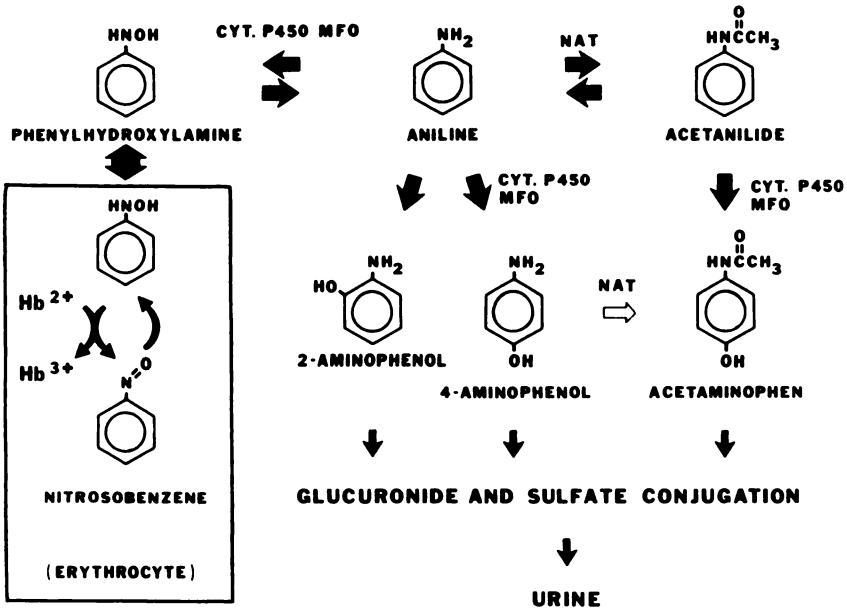

Fig. 9. Hepatic metabolism of aniline. The *inset* shows a simplified scheme of phenylhydroxylamine metabolism in erythrocytes. *CYT. P450 MFO*, cytochrome P450 mixed function oxidase; *NAT*, *N*-acetyltransferase; Hb^{2+}, hemoglobin; Hb^{3+}, methemoglobin.

hydroxylamine is present *in vivo* in sufficient quantity, and with an appropriate time course, to account for the complete methemoglobin time course after aniline. The reason the aminophenols do not contribute to methemoglobinemia in these animals is not lack of intrinsic potency, but the presence of rapid *in vivo* elimination reactions, as demonstrated by the rapid clearance of 2-aminophenol.

The finding that phenylhydroxylamine (and nitrosobenzene) mediate aniline-induced methemoglobinemia suggests that analogous arylhydroxylamines could contribute to methemoglobinemia after aniline derivatives such as dapsone, primaquine, and phenacetin. An arylhydroxylamine metabolite has been implicated in methemoglobinemia after dapsone (24). Recent studies indicate that several putative primaquine metabolites effectively stimulate methemoglobin formation *in vitro* (25, 26). However, all of the tested compounds were structural analogs of the aminophenols, and therefore may be subject to relatively rapid deactivation *in vivo*. The arylhydroxylamine derivative of primaquine, 6-methoxy-8-hydroxylaminoquinoline, generally has not been considered as a possible hemotoxic metabolite of primaquine, even though the immediate precursor to the arylhydroxylamine, 6-methoxy- 8-aminoquinoline, has been reported in erythrocytes after primaquine treatment *in vivo* (27).

The present data indicate that both N-oxidation and ring oxidation of aniline-related compounds may result in the formation of metabolic products toxic to erythrocytes. Although the mediator of aniline-induced methemoglobinemia in these rats appears to be phenylhydroxylamine (and nitrosobenzene) alone, it remains possible that the contribution of aminophenols to aniline-induced methemoglobinemia could become significant in other species or with other compounds where hepatic conjugation may occur more slowly and/or ring oxidation may be more rapid.

Acknowledgments

The authors thank Jennifer Schulte for assistance in the preparation of this manuscript.

References

1. Kiese, M. *Methemoglobinemia: A Comprehensive Treatise.* CRC Press, Cleveland (1974).
2. Brodie, B. B., and J. Axelrod. The fate of acetanilide in man. *J. Pharmacol. Exp. Ther.* **94**:29–38 (1948).
3. Glader, B. E., and M. E. Conrad. Hemolysis by diphenylsulfones: comparative effects of DDS and hydroxylamine-DDS. *J. Lab. Clin. Med.* **81**:267–272 (1973).
4. Smith, R. P., and M. V. Olson. Drug-induced methemoglobinemia. *Semin. Hematol.* **10**:253–268 (1973).
5. Kiese, M. The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. *Pharmacol. Rev.* **18**:1091–1161 (1966).
6. Kiese, M. Oxydation von Anilin Zu Nitrosobenzol in Hunde. *Naunyn Schmiedebergs Arch. Pharmacol.* **235**:360–364 (1959).
7. von Jagow, R., M. Kiese, and G. Renner. Urinary excretion of N-hydroxy derivatives of some aromatic amines by rabbits, guinea pigs, and dogs. *Biochem. Pharmacol.* **15**:1899–1910, (1966).
8. Kao, J., J. Faulkner, and J. W. Bridges. Metabolism of aniline in rats, pigs, and sheep. *Drug Metab. Dispos.* **6**:549–555 (1978).
9. Wind, M., and A. Stern. Comparison of human adult and fetal hemoglobin: aminophenol-induced methemoglobin formation. *Experientia* **33**:1500–1501 (1977).
10. Harrison, J. H., and D. J. Jollow. Rapid and sensitive method for microassay of nitrosobenzene plus phenylhydroxylamine in blood. *J. Chromatogr.* **277**:173–182 (1983).
11. Harrison, J. H., and D. J. Jollow. Role of aniline metabolites in aniline-induced hemolytic anemia. *J. Pharmacol. Exp. Ther.* **238**:1045–1054 (1986).
12. Sternson, L. A., and W. J. Dewitte. High pressure liquid chromatographic analysis of isomeric aminophenols with electrochemical detection. *J. Chromatogr.* **138**:229–231 (1977).
13. Garby, L., and P. L. Mollison. Deduction of mean red cell life span from ^{51}Cr survival curves. *Br. J. Haematol.* **20**:527–536 (1971).
14. Evelyn, K. A., and H. T. Malloy. Microdetermination of oxyhemoglobin, and sulfhemoglobin in a single sample of blood. *J. Biol. Chem.* **126**:655–662 (1938).
15. Duncan, R. C., R. G. Knapp, and M. C. Miller III. *Introductory Biostatistics for the Health Sciences.* John Wiley & Sons, New York (1977).
16. Bodansky, O. Methemoglobinemia and methemoglobin-producing compounds. *Pharmacol. Rev.* **3**:144–196 (1951).
17. Kiese, M., and M. Pekis. The reaction of *p*-aminophenol with hemoglobin and oxygen *in vivo* and *in vitro*. *Naunyn Schmiedebergs Arch. Pharmacol.* **246**:413–420 (1964).
18. Lester, D., L. A. Greenberg, and E. Shukovsky. Limited importance of methemoglobinemia in the toxicity of certain aniline derivatives. *J. Pharmacol. Exp. Ther.* **80**:78–80 (1944).
19. Smith, C. L., and D. J. Jollow. Potentiation of acetaminophen induced liver necrosis in hamsters by galactosamine. *Pharmacologist* **18**:156 (1976).
20. Parke, D. V. Studies in detoxication 84. The metabolism of ^{14}C-aniline in the rabbit and other animals. *Biochem. J.* **77**:493–503 (1960).
21. Kiese, M., and H. Uehleke. Der Ort der N-Oxydation des Anilins in bióheren Tier. *Naunyn Schmiedebergs Arch. Pharmacol.* **242**:117–129 (1961).
22. Eyer, P., H. Kampffmeyer, H. Maister, and E. Rösch-Oehme. Biotransformation of nitrosobenzene, phenylhydroxylamine, and aniline in the isolated, perfused rat liver. *Xenobiotica* **10**:499–516 (1980).
23. Heymann, E., K. Krisch, H. J. Büch, and W. Buzello. Inhibition of phenacetin- and acetanilide-induced metemoglobinemia in the rat by the carboxy-

lesterase inhibitor bis-[p-nitrophenyl] phosphate. *Biochem. Pharmacol.* **18**:801–811 (1969).
24. Kramer, P. A., B. E. Glader, and T.-K. Li. Mechanism of methemoglobin formation by diphenylsulfones: effect of 4-amino-4′-hydroxyaminodiphenylsulfone and other *p*-substituted derivatives. *Biochem. Pharmacol.* **21**:1265–1274 (1972).
25. Strother, A., R. Allahyari, J. Buchholz, I. M. Fraser, and B. E. Tilton. *In vitro* metabolism of the antimalarial agent primaquine by mouse liver enzymes and identification of a methemoglobin-forming metabolite. *Drug Metab. Dispos.* **12**:35–44 (1984).
26. Link, C. M., A. D. Theoharides, J. C. Anders, H. Chung, and C. J. Canfield. Structure-activity relationships of putative primaquine metabolites causing methemoglobin formation in canine hemolysates. *Toxicol. Appl. Pharmacol.* **81**:192–202 (1985).
27. Baty, J. D., D. A. Price Evans, and P. A. Robinson. The identification of 6-methoxy-8-aminoquinoline as a metabolite of primaquine in man. *Biomed. Mass. Spect.* **2**:304–306 (1975).

Send reprint requests to: Dr. David J. Jollow, Department of Pharmacology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425.