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The Hepatotoxicity and Urinary Metabolites of 2,4- And 2,6-Dimethylaniline in the Rat and Dog (Aromatic Amines).

Marcia L. Hardy
Louisiana State University and Agricultural & Mechanical College

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Hardy, Marcia L.

THE HEPATOTOXICITY AND URINARY METABOLITES OF 2,4- AND 2,6-DIMETHYLANILINE IN THE RAT AND DOG

The Louisiana State University and Agricultural and Mechanical Col. Ph.D. 1986

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THE HEPATOTOXICITY AND URINARY METABOLITES
OF 2,4- AND 2,6-DIMETHYLANILINE
IN THE RAT AND DOG

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences
(Toxicology Option)

by
Marcia L. Hardy
D.V.M., Louisiana State University, 1977
May 1986
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Species and isomer differences have been reported in the hepatotoxicity of 2,4- and 2,6-dimethylaniline (DMA) in the dog and rat. This study was designed to investigate the hepatotoxicity and urinary metabolites of 2,4- and 2,6-DMA in the dog and rat and the covalent binding of the two isomers in the rat as differences in metabolism may be related to the divergent hepatic responses. Male Beagles were orally dosed for 1 or 10 days with 2,6- or 2,4-DMA at 25 mg/kg. Male Fischer 344 rats were gavaged with 2,6- or 2,4-DMA at 25% of their respective LD50's (262.5 mg/kg; 117/mg/kg) for the same time period. The effects of phenobarbital (PB), 3-methylcholanthrene (3MC) and SKF-525A on the hepatotoxicity and metabolites of 2,4- and 2,6-DMA were also investigated. 24 Hr. urine samples were analysed by GC and GC/MS; liver sections were evaluated by light and electron microscopy. A final group of rats received 14C-2,4- or -2,6-DMA after a 9 day pretreatment with nonlabelled compounds. Covalent binding to hepatic DNA, RNA and protein was determined.

2,6-DMA induced hepatic fatty degeneration in all treated dogs; 2,4-DMA caused no detectable lesions. In rats 2,6-DMA produced no significant liver lesions, whereas 2,4-DMA induced hepatomegally and a subtle but distinct change characterized by swollen cells with voluminous homogeneous cytoplasm and segregation or clumping of cytoplasmic substructures. Concurrent treatment with PB and 2,4-DMA resulted in
death of 50% of the animals by Day 5. 3MC treatment enhanced the 2,4-DMA lesion. 2,4-DMA alleviated an SKF-525A-induced midzonal fatty degeneration of the liver, whereas 2,6-DMA was less effective in this regard.

The major urinary metabolite of 2,4-DMA in the dog was 6-hydroxy-2,4-DMA. Minor metabolites included 4-amino-3-methyl-benzoic acid and N,2,4-trimethylaniline. The major urinary metabolite of 2,6-DMA was 4-hydroxy-2,6-DMA. Minor metabolites included 2-amino-3-methyl-benzoic acid, N,2,6-trimethylaniline, 2,6-dimethylnitrosobenzene, 4-imino-3,5-dimethyl-quinone, and the glycine conjugate of the benzoic acid. Rats produced N-acetyl-4-amino-3-methyl-benzoic acid as the major metabolite of 2,4-DMA and N,2,4-trimethylaniline in small amounts. 2,6-DMA was primarily excreted as 4-hydroxy-2,6-DMA; N,2,6-trimethylaniline was a minor metabolite.

The covalent binding of 14C-2,4-DMA to rat hepatic DNA, RNA or protein was significantly greater than that of 14C-2,6-DMA. The above results appear to indicate that differences in metabolism, with possible activation to reactive metabolite(s), are important in the divergent hepatic effects of 2,4- and 2,6-DMA in the rat and dog.
INTRODUCTION

Aromatic amines show great diversity of structure, and because of this diversity, are quite useful compounds. Each is composed of one or more benzene rings to which at least one amine (\(-\text{NH}_2\)) group is attached. In primary aromatic amines the nitrogen is solely substituted by hydrogen; aniline (\(\text{C}_6\text{H}_5\text{NH}_2\)) is a primary aromatic amine. In secondary amines one of these hydrogens is substituted by alkyl or aryl groups; in tertiary aromatic amines both hydrogens on the nitrogen are substituted. The benzene ring may also be substituted as in toluidine where a methyl group replaces a hydrogen atom, conjoined as in 4,4'-diaminodiphenyl, bridged as in 4,4'-methylene-bis-dianiline or condensed as in 1-naphthylamine.¹ (Figure 1.)

![Structures of some aromatic amines.](image)

Figure 1. Structures of some aromatic amines.

¹
A great number of compounds are thus possible; in theory infinite, in practice enormous. This, coupled with their ability to react with other amines and chemical classes, makes aromatic amines very valuable in organic synthesis, particularly in the manufacturing, pharmaceutical and chemical industries. These compounds are used as synthetic dyestuffs of cloth, fur, hair, paper and paint, as antioxidants in rubber, as curing agents for polyurethane plastics and as the foundation in the synthesis of a number of drugs.2

Interest in the toxicity of aromatic amines has grown as their industrial use has expanded. It was early recognized that aniline could cause cyanosis. The cause of this cyanosis was later found to be methemoglobin formation.3 This in turn led to the discovery that the methemoglobin production was not due to aniline itself, but was due primarily to phenylhydroxylamine, a metabolite of aniline.4 It was also noted that the induction of methemoglobin was not limited to aniline alone; other aromatic amines were capable of producing this response. Certain other acute toxicities were also reported early in the use of these compounds i.e., their ability to induce skin sensitization and, after massive exposure, severe hematuria.3

The more serious chronic effects of aromatic amines were not evident until they had been in use for approximately 30 years. In 1895 a German surgeon, Rehn, reported three cases of bladder tumors in German dyestuff workers.5 This was a relatively uncommon disease and the three cases proved an unusually high incidence. Rehn
attributed the bladder tumors to aniline exposure and the term "aniline tumore" was used for many years. The term may be a misnomer as these tumors were later attributed to 2-naphthylamine and benzidine exposure. While bladder tumors can be induced by these two amines, there is no definite evidence that aniline was not involved. Concern for the carcinogenic risk posed by aromatic amines expanded work in this area and by 1979, when the Occupational Safety and Health Administration recognized 17 carcinogens, 9 were aromatic nitro and amino compounds (2-acetylaminoflourine, 4-aminobiphenyl, benzidine, 3,3'-dichlorobenzidine, 4-dimethylaminoazobenzene, 1-naphthylamine, 2-naphthylamine, 4,4'-methylene-bis-(2-chloroaniline), and 4-nitrophenyl).

The acute effect of methemoglobinemia and the serious chronic effect of cancer induction created interest in the toxicity of the aromatic amines as a family. Certain of these compounds, such as aniline, benzidine and 2-acetylaminoflourine, have been investigated extensively. Others have received little attention despite increasing use. The dialkylanilines and methylene bridged anilines enjoy extensive use, yet little information on their toxicity is available in the open literature. In addition, the information available is often of limited use because of failure to clearly specify the compound under study. For example, reports on the toxicity of 'dimethylaniline' could refer to 2,4-dimethylaniline, 2,6-dimethylaniline, N,N-dimethylaniline, or any other combination. The toxic effects of these compounds are not necessarily the same,
even though they are all isomers of 'dimethylaniline'. Thus, much of the older literature is of little value.

The purpose of this study is to investigate the hepatotoxicity, macromolecular covalent binding, and metabolism of 2,4- and 2,6-dimethylaniline in the rat and dog. These two compounds differ from one another structurally by the position of one methyl group; yet one is more acutely hepatotoxic to the dog; the other to the rat. Furthermore, the histopathological effect produced by each isomer is different in the two species. One has induced fatty change in the dog; the other hepatomegaly, hepatocytic vacuolization and bile duct dilation in the rat. The dose required to produce these changes is also significantly lower in the dog than the rat. In addition, 2,6-dimethylaniline has induced tumors in the nasal turbinates of treated rats, whereas the carcinogenic response to 2,4-dimethylaniline was equivocal. Despite these interesting phenomena, very little published work exists on the toxicity of these compounds.

The answer to the divergent responses produced by the two isomers may be related to differences in metabolism. Biotransformation of the two isomers may proceed via separate routes as the result of either differences in chemical structure or species variation in metabolism. Differences in biotransformation could, in turn, affect the production of reactive chemical species, which may bind to critical macromolecules, resulting in either target organ toxicity or carcinogenesis.
Finally, 2,4- and 2,6-dimethylaniline are the simplest of the dialkylanilines and are intermediate in structural complexity between aniline, the bridged anilines and the complex multiple ring aromatic amines. Understanding the toxic effects and metabolism of these simple aromatic amines may allow prediction of the toxicity of similar compounds and their more complex congeners, and direct the synthetic chemist to the production of compounds which, while effective for their intended purpose, are also relatively nontoxic to humans under potential threat of exposure.
The purpose of this study is to investigate the hepatotoxicity, covalent binding and metabolism of the dialkylaniline isomers 2,4- and 2,6-dimethylaniline. Traditionally, an historical review would be confined to these two compounds. Unfortunately, published material in this area is meager and such an isolated review would fail to demonstrate the relationship between these and other aromatic amines. Therefore, this review is divided into five subject areas: aniline, o-toluidine, 2,4-dimethylaniline, 2,6-dimethylaniline and selected methylene bridged aromatic amines. This is a logical progression from the simplest aromatic amine, aniline, to those of more complex structure. Hopefully, by proceeding in this manner, a more thorough understanding of the subject material will be achieved.

**Aniline**

![Aniline](image)

Figure 2. Aniline.

Aniline, the simplest aromatic amine, is an oily, lipid soluble liquid. It is used in the production of dyestuffs, rubber accelerators and antioxidants and is an intermediate in the manufacture of pharmaceuticals, photographic developers, plastics and ion exchange resins. It is readily absorbed after inhalation,
ingestion or skin contact. Aniline easily penetrates clothing and leather gloves and boots, and cases of industrial intoxication have resulted from prolonged contact through these articles of apparel. In both man and animals, its most outstanding acute toxic effect is the production of methemoglobin. The formation of methemoglobin is the result of oxidation of hemoglobin; primarily by the N-oxidation product of aniline, phenylhydroxylamine. Variation in species susceptibility to this effect is quite marked, with the cat being very sensitive and the rat relatively resistant. The order of susceptibility to the production of methemoglobin is reported to be as follows: cat>man>dog>rat>rabbit and monkey.

Aniline is not the only substance capable of inducing methemoglobin; nitrates, chlorates, phenacetin, acetanalide, sulfonilamide, nitrobenzene and quinones also produce this effect. Furthermore, under normal cellular conditions erythrocytes contain small amounts of methemoglobin due to the spontaneous oxidation of hemoglobin. To reduce this naturally occurring methemoglobin, erythrocytes contain two enzyme systems; methemoglobin diaphorase (diaphorase I) and methemoglobin reductase (diaphorase II). Reducing equivalents for these enzymes are derived from NADH and NADPH, respectively. These enzyme systems reduce spontaneously formed methemoglobin as well as that induced by xenobiotics.

The induction of methemoglobin does not involve the formation of a covalent bond with hemoglobin; it is due to a cyclic process of
oxidoreduction that occurs in the red blood cell. In the case of aniline, the induction of methemoglobinemia appears to begin with its metabolism to small amounts of phenylhydroxylamine by the liver. Erythrocytes passing through the hepatic sinusoids trap minute amounts of the metabolite and, in the presence of oxygen, oxidize phenylhydroxylamine to nitrosobenzene. Simultaneously, hemoglobin (Fe++) is oxidized to methemoglobin (Fe+++). Nitrosobenzene either a) is reduced by methemoglobin reductase and NADPH to phenylhydroxylamine, which can then oxidize another molecule of hemoglobin, b) forms labile adducts with glutathione or c) forms non-labile adducts with reactive SH-groups of hemoglobin. A small amount of nitrosobenzene is reduced to its parent amine, which eventually terminates the reaction. (Figure 3.) Contributing to the cyclic nature of methemoglobin formation is the high affinity of the nitroso derivative for methemoglobin reductase; binding between the two inhibits the enzyme and prevents the reconversion of methemoglobin to hemoglobin.

Acute exposure to aniline at levels high enough to produce a significant degree of methemoglobinemia results in cyanosis, tachycardia, tremors, collapse, coma and potentially, death. Sub-chronic exposure in rats has produced anemia, splenic enlargement, hemosiderosis and sinusoidal engorgement, and bone marrow hyperplasia. These effects are thought to be due to aniline-induced damage to red blood cells which in turn increases splenic scavaging. Whether or not this damage is related to the formation of methemoglobin has yet to be determined.
Figure 3. Cyclic process of oxidoreduction in the red blood cell.
In a study in which rats were treated orally for 1 or 10 days with $^{14}$C-aniline HCl, it was found that radioactivity was concentrated in erythrocytes rather than in the plasma. Gralla et al. also reported the same observation. Furthermore, rats treated for 10 days had a 12.3-fold greater concentration of radioactivity in the spleen 24 hr after the last dose than rats receiving a single dose. Radioactivity in whole blood, plasma, liver, kidney, brain, lung, heart and fat increased only 1.8 to 3.8 times in 10-day compared to 1-day treated rats. This appears to indicate a splenic accumulation of isotope with repetitive dosing. Since one of the normal functions of the spleen is the removal of damaged or abnormal erythrocytes, this accumulation may be mediated by splenic scavaging of erythrocytes containing elevated concentrations of aniline and its metabolites. Anemia, splenic enlargement, hemosiderosis and engorgement, and bone marrow hyperplasia would be logical consequences of accelerated splenic scavaging.

The spleen and bone marrow appear to be the major sites affected by aniline following sub-chronic exposure. Short et al. found that aniline administered orally at 110 mg/kg (25% of the LD$_{50}$) for 5, 10 or 20 days produced no evidence of adverse effects in the liver, kidney, urinary bladder, thyroid, trachea or esophagus in the male Fischer 344 rat on histopathologic examination. Examination of the spleen and bone marrow revealed the previously reported pathology, i.e., increased weight, engorgement and congestion of the spleen, extramedullary splenic hematopoiesis, hemosiderin accumulation and marrow hypercellularity.
Gralla et al. fed aniline at 10, 30, 100, 300 or 1000 mg/kg/day for 1 year to Fischer 344 rats of both sexes. The lowest dose caused little toxic response at the end of one year, but each higher dose produced changes in the spleen in a dose- and time-related manner. The spleen was grossly enlarged and the capsule was diffusely thickened. Bands of hyperplasia extended centrally into the parenchyma. The red pulp was hyperplastic and showed signs of increased hematopoiesis. Other changes included methemoglobinemia, reticulocytosis, Heinz body formation, anemia and bone marrow erythroid hyperplasia.

Aniline has undergone extensive mutagenicity testing. It was reported to be negative on the Ames test, both with and without Arochlor 1254-induced microsomal activation in Salmonella typhimurium strains TA 1535, TA 1536, TA 1537, TA 1538, TA 98 and TA 100.\textsuperscript{17,18,19,20} Aniline was also non-mutagenic in normal and DNA polymerase deficient Escherichia coli\textsuperscript{18} and lacked recombinogenic activity in Saccharomyces cerevisiae.\textsuperscript{21} Parodi et al., however, using the alkaline elution assay found aniline to be capable of inducing DNA damage in rat liver and kidney, and also to be capable of causing sister chromatid exchange \textit{in vivo} in mice.\textsuperscript{22} On the other hand, Lutz estimated a covalent binding index for aniline and judged it to be extremely low.\textsuperscript{23} Covalent Binding Index or CBI is defined as DNA binding per dose, in the units:
micromoles chemical bound/mole nucleotide

mmole chemical adm./kg body weight,

and is suggested by its author to be an estimation of the potency of a genotoxic chemical carcinogen.\(^{23}\)

Despite its general lack of mutagenicity/genotoxicity in traditional in vitro tests, aniline has been found to be carcinogenic in male and female Fischer 344 rats.\(^{24}\) Fischer 344 rats and B6C3F1 mice were fed aniline at 1.3 or 1.6 percent and 0.6 or 1.2 percent, respectively, of the diet for 103 weeks. There was no evidence of compound-induced carcinogenicity in B6C3F1 mice of either sex, but splenic hemangiosarcomas, fibrosarcomas and sarcomas NOS as well as a combination of fibrosarcomas and sarcomas NOS of multiple body organs were found in male and female rats.

The difference in susceptibility to aniline induced carcinogenicity in rats and mice may be due to differences in distribution or metabolism in the two species. McCarthy et al. found major differences in metabolites and binding potential of aniline in rats and mice.\(^{25}\) As compared with a single dose of labeled compound, pretreatment with unlabeled aniline for 7 days caused a decrease in binding of \(^{14}\)C-label to RNA and protein of various tissues in mice, whereas the same treatment regimen brought about an increase in binding to RNA and protein in the rat. Binding to rat splenic macromolecules increased to a particularly large degree when compared to other tissues. There was no significant binding to DNA in either
species. These findings were coupled with cytochrome P-450 induction in pretreated mice (but not in rats), a significantly higher $V_{\text{max}}$ and lower $K_m$ for aniline p-hydroxylase in mice over rats, and no difference in the $K_m$ or $V_{\text{max}}$ of N-hydroxylase in the two species. The higher $V_{\text{max}}$ in mice would allow these animals to convert aniline to the p-hydroxylated product at a faster rate than that of rats; however, the substrate concentration at which saturation is reached would be lower in mice than rats. The decrease in binding to RNA and protein in pretreated mice may be a reflection of cytochrome P-450 induction. It is possible that a detoxification pathway, as opposed to an activation pathway, was induced.

A substantial difference in metabolic profiles of urinary metabolites was also seen between the two species in this study. Of aniline compounds present in the urine of treated rats, the unchanged parent compound was isolated in greatest quantities followed (in order of decreasing amounts) by the sulfate conjugates of 4-hydroxyacetanilide, p-aminophenol and o-aminophenol. A small amount of the glucuronide conjugate of 4-hydroxyacetanilide was also isolated. Sulfate conjugates represented the greatest fraction of urinary metabolites. In a separate study, Bus et al. also isolated a preponderance of sulfate conjugates from aniline-treated rats. 4-Hydroxyacetanilide sulfate was the urinary metabolite present in greatest amounts with only a small fraction of the dose eliminated as unchanged aniline. Sulfate esters of p-aminophenol, o-aminophenol, acetanilide and 2-hydroxyacetanilide were also
isolated as were small amounts of the glucuronide conjugates of aniline, acetaminophen, p-aminophenol, o-aminophenol and 2-hydroxyacetanilide. Low levels of free acetanilide and acetaminophen were also identified. (Figure 4.)

McCarthy et al. reported a substantially different profile for metabolites in mouse urine. The glucuronide conjugate of 4-hydroxyacetanilide was the metabolite present in greatest amounts. Roughly equal quantities of aniline and o-aminophenol sulfate were isolated. Other sulfate esters included 2-hydroxyacetamide and p-aminophenol. p-Aminophenol was also present as a glucuronide conjugate. (Figure 5.)

In summary, the major differences in urinary metabolites between the rat and the mouse were the extent of sulfation vs. glucuronidation of 4-hydroxyacetanilide and the overall preference of sulfation as a route of conjugation in the rat vs roughly equal concentrations of glucuronide and sulfate esters in the mouse. The McCarthy study on the species differences in aniline metabolism must be criticized for lack of uniformity in dosage regimens. On a mg/kg basis, mice received a much higher dose of aniline than did rats. If the higher dose was sufficient to saturate the sulfate conjugation pathway or to deplete the sulfate pool in mice, a shift to glucuronide conjugation could have occurred.
Figure 4. Proposed pathway of aniline metabolism in the rat, based on urinary metabolites. (*Major urinary metabolite.)
Figure 5. Proposed pathway of aniline metabolism in mice, based on urinary metabolites. (*Metabolites present in greatest quantities.)
The difference in carcinogenicity between the two species could have been related to the decline in binding to cellular macromolecules which in turn may have been related to differences in metabolism of aniline. With respect to binding, mice appeared to be more efficient in metabolizing aniline than rats and this efficiency increased with duration of exposure to aniline. Rats, on the other hand, tended to increase production of reactive metabolites with continued exposure as evidenced by increased binding in the spleen, the target organ. Rats also favored sulfation as the major conjugation pathway. As the availability of sulfate \textit{in vivo} has been shown to be limited in other studies, chronic aniline exposure could deplete the sulfate pool and alter metabolic pathways; possibly towards the formation of reactive products.

A study in which $^{14}$C-aniline HCl was administered on an acute and chronic basis tends to argue against sulfate depletion as the basis for reduced efficiency of metabolism in rats.\textsuperscript{14} The tissue distribution of radioactivity was similar after oral doses of 10, 30, or 100 mg/kg $^{14}$C-aniline HCl. Peak tissue levels were seen 0.5 and 2.0 hr after dosing and were followed by a rapid biphasic elimination of radioactivity. Excretion of radioactivity was essentially complete 24 hr after all doses, with the majority of excreted radioactivity being recovered in the urine. Feeding aniline HCl in the diet at 100 mg/kg/day for 1 or 4 weeks did not alter the plasma disposition of $^{14}$C-activity following a single 100 mg/kg oral dose of $^{14}$C-aniline HCl. Urinary metabolites were also essen-
tially unchanged after 1 or 4 weeks of treatment. After 1 week of administration, 40.6% of the dose was excreted as sulfates, 3.9% as glucuronides, and 3.5% unconjugated. At the end of 4 weeks of treatment, 39.1% of the dose was recovered as sulfate conjugates, 8.4% as glucuronides and 4.9% as unconjugated metabolites. Sulfates of acetaminophen and p-aminophenol accounted for most of the sulfate metabolite fraction after 1 or 4 weeks of administration. Thus, repeated exposure of rats to aniline HCl did not appear to result in any significant alteration in urinary metabolites or excretion rate. Unfortunately, a similar study using mice as the experimental animal is not available in the literature. As repetitive administration of aniline in this species increases hepatic cytochrome P-450 content, it is possible that urinary metabolites and/or elimination rate are altered in the mouse on chronic exposure.

In contrast to the similar plasma disposition and metabolite pattern after 1 and 4 weeks of treatment, oral administration of 100 mg/kg of 14C-aniline HCl to rats for 3 or 10 days significantly altered the tissue distribution of radioactivity compared to a single dose. Radioactivity was increased in the spleen by 4.7- and 12.3-fold after 3 and 10 days of treatment, respectively. Radioactivity increased 1.7- to 2.6-fold after 10 days in the liver, lung, heart, brain, fat, kidney, plasma and erythrocyte. Equilibrium concentrations in tissues other than the spleen were reached in 3 days. The increase in radiolabel in the spleen was not merely due to an accumulation of radioactivity over time, as
pretreatment with unlabelled aniline followed by a single dose of 
$^{14}$C-aniline also increased radioactivity in the spleen.

The fact that urinary metabolites and elimination rates were not significantly altered after chronic aniline administration does not necessarily mean that a minor metabolic pathway was not altered or that minute quantities of a 'new' reactive product were not produced. Small quantities of a highly reactive product, transported to the spleen via the red blood cells and not detectable in urine could account for the carcinogenicity. Alternatively, a detoxification pathway, i.e. erythrocytic glutathione, could be depleted on chronic exposure or serve as a transport mechanism to the target organ, the spleen. It is possible that the spleen itself may biotransform aniline or one of its metabolites to a reactive species. As aniline has a very low DNA covalent binding index and yet binds extensively to protein and RNA, it may act as a carcinogen via an epigenic mechanism. Or, perhaps the carcinogenicity may be mediated by the increased physiological demands placed on the spleen as it scavenges damaged erythrocytes and is therefore unrelated to the presence of aniline and/or its metabolites.


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o-Toluidine

![Chemical Structure](image)

Figure 6. o-Toluidine.

Toluidine (mono-methylaniline) differs from aniline by the substitution of a methyl group for a ring hydrogen. The methyl group may be located ortho, meta or para to the amine function. Mutagenicity and carcinogenicity vary between the three isomers. This review will center on o-toluidine as it shows the greatest toxic potential of the three.

o-Toluidine and its hydrochloride salt are used as intermediates in the manufacture of a large number of textile dyes.\(^2\)\(^6\) It is also used as a photographic dye, a reagent in a clinical assay for glucose and hemoglobin, and as an antioxidant in the manufacture of rubber.\(^2\)\(^7\),\(^2\)\(^8\),\(^2\)\(^9\)

Clinical signs of acute toxicity are very similar to those of aniline and include the formation of methemoglobin and subsequent cyanosis. The induction of methemoglobin by o-toluidine has been reported in man, rats, and mice.\(^3\)\(^0\),\(^3\)\(^1\),\(^3\)\(^2\) Hematuria after massive exposure has been reported in man.\(^3\)
Lesions were observed in the spleen and bone marrow in a subchronic study in which male Fischer 344 rats were gavaged with o-toluidine for 5, 10, or 20 days at 225 mg/kg/day (25% of the LD$_{50}$). A significant increase in splenic congestion of treated rats was seen, characterized by engorgement of the sinusoids of the red pulp with erythrocytes. Splenic hemosiderosis was marked and a significant increase in spleen weight occurred. Hematopoiesis was increased in the spleen, and the bone marrow showed an increase in cell density. The myeloid/erythroid ratio in bone marrow was considered within normal limits, however. No significant changes were seen in liver, thyroid, kidney, urinary bladder, trachea or esophagus. This pattern of lesions was essentially the same as that induced in aniline-treated rats included in the same study and was probably related to a toxic effect on the red blood cell.

o-Toluidine also reacts very similarly to aniline on in vitro mutagenicity tests. Like aniline, it was non-mutagenic toward Salmonella typhimurium with and without Arochlor 1254-induced S-9 activation. Bacterial strains tested were TA 1535, TA 1537, TA 1538, TA 98 and TA 100. It was also negative for recombinogenic activity in Saccharomyces cerevisiae D3 both with and without Arochlor 1254-induced microsomes. o-Toluidine did show positive activity toward Escherichia coli (pol A-) strain. This strain of E. coli lacks DNA polymerase I, an enzyme which functions in DNA repair. Normal (pol A$^+$) E. coli did not display mutagenesis when exposed to o-toluidine.
When subjected to a National Cancer Institute (NCI) bioassay, o-toluidine was judged to be carcinogenic. This situation is very similar to that of aniline where in vitro mutagenicity tests also failed to reveal conclusively positive results, and yet long-term feeding trials produced an increased incidence of tumors. In the NCI bioassay male and female Fischer 344 rats and B6C3F1 mice were fed o-toluidine hydrochloride in the diet at 3000 or 6000 ppm and 1000 or 3000 ppm, respectively, for 101 to 104 weeks. In rats, the compound induced several types of sarcomas in the spleen in both males and females, mesotheliomas of the abdominal cavity or scrotum in males, and transitional cell carcinomas of the urinary bladder in females. There was also an increased incidence of fibromas of subcutaneous tissues in males and fibroadenomas or adenomas of the mammary gland in females. Male mice had hemangiosarcomas at various sites and females had hepatocellular carcinomas or adenomas. In similar long term feeding studies, the same organ pattern of tumors resulted; that is, urinary bladder tumors and subcutaneous fibromas or fibrosarcomas in rats and vascular tumors in mice. The primary difference in tumor induction between these studies was that the bladder cancers were noted in male rats in the earlier studies, whereas they were found in female rats in the NCI bioassay.

In comparing the NCI bioassays of aniline and o-toluidine, close similarities emerge. Both aniline and o-toluidine induced splenic sarcomas and fibrosarcomas in various organs of rats. o-Toluidine,
however, produced bladder tumors in female rats while no such effect was observed for aniline in either male or female rats. Furthermore; aniline, at much higher doses than o-toluidine, had no carcinogenic effect in B6C3F1 mice. o-Toluidine was judged to be carcinogenic in this strain of mice.

Aniline and o-toluidine differ from one another structurally by the presence or absence of one methyl group. They both produce similar acute and subchronic toxicities. They both induce a similar pattern of tumors in rats, with the exception of bladder tumors in the case of o-toluidine. They markedly diverge in tumor incidence in mice - aniline was non-carcinogenic whereas o-toluidine was judged to be carcinogenic. Furthermore; the tumors induced in mice by o-toluidine were of a different cell type than those in rats - hemangiosarcomas vs fibrosarcomas. Perhaps their similarities and disimilarities may be explained on the basis of metabolism.

Son et al. reported an elegant study on the metabolism of $^{14}$C-labeled o-toluidine in Fischer 344 rats. Forty-eight hours after treatment, 83.9% of the $^{14}$C-label was recovered in the urine, 3.3% in the feces, and 1.4% in exhaled air. The majority of urinary metabolites (51% of the dose) were conjugated with either sulfate or glucuronic acid. Approximately 23% of the total identified urinary metabolites were acetylated, 66% were sulfate conjugates and 11.6% were glucuronide conjugates. The sulfate conjugates identified were 4-amino-m-cresol (27.8% of the dose), N-acetyl-4-
amino-m-cresol (8.5%), and 2-amino-m-cresol (2.1%). Glucuronide conjugates accounted for 6.7% of the dose and were glucuronides of 4-amino-m-cresol (2.6%), N-acetyl-4-amino-m-cresol (2.8%) and N-acetyl-o-aminobenzyl alcohol (1.3%). Non-conjugated metabolites were o-toluidine (5.1%), azoxytoluene (0.2%), o-nitrosotoluene (0.1%), N-acetyl-o-toluidine (0.2%), N-acetyl-o-aminobenzyl alcohol (0.3%), 4-amino-m-cresol (1.6%), N-acetyl-4-amino-m-cresol (0.3%), anthranilic acid (0.3%) and N-acetylanthranilic acid (0.3%). (Figure 7.) N-acetylation and hydroxylation at the 4 position of o-toluidine predominated. Minor metabolic pathways included hydroxylation at the 6 position, oxidation of the methyl group and oxidation of the amino group. Sulfate conjugates predominated over glucuronides by a ratio of 6:1.

Like aniline, o-toluidine is primarily N-acetylated, p-hydroxylated and conjugated with sulfate in the rat. The para-position is the preferred site of oxidation with approximately 42% of the dose of o-toluidine in the form of para-hydroxylated products and only 2% as ortho-hydroxylated product. In the rat, rabbit, pig and sheep, aniline is also hydroxylated preferentially in the para-position as compared to the ortho site. Interestingly, this same preference for oxidation para to the amine function holds true with the N-acetylated derivatives of o-, m-, and p-toluidine in the rabbit. The major urinary metabolites of o- and m-acetotoluidine are 6-amino-m-cresol and 5-amino-o-cresol, respectively. p-Acetotoluidine is virtually completely oxidized to p-acetamidobenzoic acid. Oxidation of the methyl group to a benzoic
Figure 7. Metabolism of o-toluidine in the rat. Brackets indicate proposed pathway. From Son, O. S. et al.
acid derivative increases with increasing distance between it and the amine function (p>m>o). Unfortunately, there are no comparable studies of o-toluidine in mice in the literature.

2,4-Dimethylaniline

![Figure 8. 2,4-Dimethylaniline.](image)

2,4-Dimethylaniline (2,4-DMA, 2,4-m-xylidine, 2,4-dimethyl-benzylamine), an oily aromatic liquid, is used commercially as a dye and polyurethane intermediate and has been recovered as a metabolite of food dyes, cosmetics and pharmaceuticals. This compound has not undergone the extensive testing of aniline or o-toluidine; however, the literature shows that it differs markedly in toxicity from the simpler compounds. The methemoglobinemia and splenic lesions seen with aniline and o-toluidine were not characteristic of 2,4-DMA treated animals. Hepatotoxicity was the predominant lesion, but in this regard, 2,4-DMA treatment resulted in marked species variation in the type and degree of toxicity induced. The compound has been shown to produce definite degenerative lesions in the rat, but produced only a mild degree of fatty degeneration in the livers of treated dogs.

Fischer 344 rats treated for 5, 10 or 20 days with 117 mg/kg/day of 2,4-DMA (25% of the LD50) developed a toxic hepatopathy characterized by extensive cloudy swelling, diffuse hepatocellular
necrosis, early periacinlar connective tissue proliferation, biliary hyperplasia and periacinlar vacuolar degeneration. Liver weights were significantly increased. No lesions were observed in the spleen, kidney, bone marrow, thyroid, trachea or esophagus.

Earlier studies produced much the same results. Lindstrom et al. in 1963 fed Osborne-Mendel rats 2,4-DMA for 3 or 6 months at 375, 750, 2500, 5000 or 10000 ppm in the diet. A significant decrease in weight gain was noted for male and female rats at 3 and 6 months for the 3 highest doses. At 6 months there was also a significant increase in liver and kidney weights for both sexes at all dose levels. Splenic weight increased over control values for the two highest doses, and testicular weight was increased at the 3 highest doses. Heart weight was significantly increased in male rats in the 10000 ppm group. This group also had scattered foci of cholangiofibrosis, new bile duct formation, and foci of hepatic cellular hyperplasia. In addition, the high dose group also showed slight renal damage characterized by tubular atrophy, interstitial fibrosis, chronic inflammation, and various papillary changes. Doses lower than 10000 ppm produced little or no renal damage. The spleen, glandular stomach, small intestine, colon, adrenal, pancreas and testes were all negative with respect to histopathologic changes.

Magnusson et al. treated Sprague-Dawley rats with 2,4-DMA at 20, 100 or 500-700 mg/kg daily for 4 weeks. Liver weights were
increased at all doses and necrosis and vacuolization was observed in the high dose rats. A slight proliferation of bile ducts was seen. No fatty change was found. The kidney, the only other organ to be examined histologically, was normal. In 1979 Magnusson published a second paper on the hepatic effects of 2,4-DMA in which he characterized the liver lesions by ultrastructural, histochemical and biochemical means. Male and female Sprague-Dawley rats were treated with 2,4-DMA at 400-500 mg/kg/day for 4 weeks. Livers were enlarged, but otherwise appeared normal to the unaided eye. An apparent increase in size of individual hepatocytes and occasional isolated necrotic cells were found on histopathological examination. Isolated hepatic cells contained vacuoles and inclusion bodies. Dilated bile duct canaliculi were also seen. Staining with PAS showed a centrilobular decrease in liver glycogen. Glucose-6-phosphatase activity was also decreased in the centrilobular region. 2,4-DMA treatment produced an increase in smooth endoplasmic reticulum, microsomal protein and cytochrome P-450 content. Aniline hydroxylase and glucuronyltransferase activities were also stimulated. In another study, Gopinath et al. treated male and female Charles River CD rats with 2,4-DMA at 400 mg/kg/day for 7 days. Histopathologic examination of the liver showed bile duct hyperplasia, liver cell enlargement, liver cell necrosis, biliary canalicular dilation and proliferation of the endoplasmic reticulum. Serum glutamic dehydrogenase and glutamate pyruvic index of liver damage, were elevated.
Concurrent with his 1971 study of the hepatotoxic effects of 2,4-DMA in rats, Magnusson also evaluated the effect of 2,4-DMA in dogs. Male and female beagles received oral doses of 2, 10 or 50 mg/kg daily for 4 weeks. The dogs receiving the highest dose vomited intermittently shortly after administration of the compound. These dogs also developed a modest degree of liver enlargement, and mild fatty degeneration was found on examination of histologic sections. No hepatic lesions were reported for doses of 2 or 10 mg/kg/day. The kidneys were normal for all doses.

In summary, the addition of one methyl group on the benzene ring, as in 2,4-DMA, produced a substantially different form of toxicity than aniline or o-toluidine. Following aniline or o-toluidine administration, lesions predominated in the spleen and bone marrow and were probably related to a primary effect on the red blood cell. 2,4-DMA pathology, however, was confined to the liver. Not only were the lesions different with 2,4-DMA, but an apparent species difference in toxic effects was also seen. Dogs exhibited only a slight degree of fatty degeneration, while a greater degree of liver enlargement, vacuolization and biliary proliferation was produced in rats.

Two studies on the metabolism of 2,4-DMA were available in the literature. A 1961 report by Lindstrom identified the metabolites of 2,4-DMA in the rat using paper chromatography. Male Osborn-Mendel rats were dosed orally at a rate of 200 mg/kg/day for an unstated length of time. 2,4-DMA metabolites recovered from urine
included N-acetyl-2,4-DMA, 2,4-DMA sulfate, N-acetyl-3-methyl-4-amino-benzoic acid, 3-methyl-4-amino-benzoic acid and the glycine conjugate of 4-amino-benzoic acid. Lindstrom's findings indicated that the primary route of 2,4-DMA metabolism was oxidation of the para methyl group to form 3-methyl-4-amino-benzoic acid. Small amounts of this metabolite were excreted unchanged or as the glycine conjugate, but the largest fraction was excreted as N-acetyl-3-methyl-4-amino-benzoic acid. No free (non-conjugated) hydroxylated metabolites were detected. (Figure 9.)

Lindstrom's study showed that 2,4-DMA followed the same general pattern of metabolism as aniline and o-toluidine i.e., that of oxidation in the para position. In this case, the benzoic acid derivative was produced as the para position of 2,4-DMA was occupied by a methyl group. In contrast to aniline and o-toluidine, sulfate conjugation was not the primary route of excretion and N-acetylation took on a more dominant role.

The above findings were not surprising considering the preference in rodents and rabbits for hydroxylation para to the nitrogen in the two simpler aromatic amines. This preference was still demonstrated in the metabolism of 2,4-DMA by the rat, but in this case the para position was occupied by a methyl group. Oxidation of this group, by necessity, must produce either the alcoholic or carboxylic derivative. Sulfate conjugation in this position, dominant in the metabolism of aniline and o-toluidine, could only occur with demethylation followed by hydroxylation and conjugation. Ring demethylation
Figure 9. Structures of the major and minor metabolites of 2,4-DMA isolated by Lindstrom et. al. from the urine of rats.
is an extremely unlikely, if not impossible, event. It is possible that oxidation could occur in the unoccupied 6 position (ortho to the nitrogen), but this appears unlikely considering the low extent of ortho-hydroxylation of aniline in the rat, rabbit, mouse, pig and sheep and of o-toluidine in the rat. Urinary metabolites of 2,4- DMA in the dog, however, may be quite different from those in the rat as carnivores are reported to preferentially oxidize aniline in the ortho-, rather than the para-, position.49

In 1983 Nohmi reported the isolation of N-hydroxy-2,4-DMA from an in vitro system using liquid chromatography and mass spectrometry.50 Male Fischer rats were pretreated with polychlorinated biphenyls, sacrificed, and an S-9 fraction (9000 xg supernatant) was isolated. 2,4-DMA and the cofactors glucose-6-phosphate, NADPH, NADH and magnesium chloride were added to the test system and incubated for 30 minutes. Several metabolites were isolated on reverse-phase liquid chromatography, but only N-hydroxy-2,4-DMA was identified. Approximately 0.57% of 2,4-DMA was converted to the N-hydroxylated metabolite.

Using an Arochlor 1254 induced hepatic S-9 fraction, in vitro Salmonella mutagenicity tests showed that 2,4-DMA required metabolic activation to be mutagenic.19,51 Bacterial strains tested included TA 1535, TA 1537, TA 1538, TA 98, and TA 100. No mutagenic effect was seen when the S-9 fraction was deleted. Nohmi, in his metabolism study on 2,4-DMA, found that the mutagenic metabolite of
2,4-DMA was the N-hydroxy derivative. Metabolites were isolated from an in vitro system, tested for their ability to mutate Salmonella strains, and identified by mass spectrometry. However, 2,4-DMA was negative when tested for mutagenesis in the alkaline dilution/DNA breakage test in Chinese hamster lung fibroblasts with Arochlor induced S-9 activation.

Only one long term feeding study designed to investigate the carcinogenicity of 2,4-DMA was found in the literature. 2,4-DMA increased the incidence of lung tumors in female CD-1 mice when fed at a level of 250 ppm in the feed for 18 months. No such effect was observed in females treated at a feed concentration of 125 ppm or in males treated at either of the two levels. Concurrent feeding of male Charles River CD rats at varying levels (250 ppm to 4000 ppm) for varying lengths of time also produced no carcinogenic effect. It was concluded that the compound was "not a very active carcinogen" and that the results may be somewhat ambiguous as only one sex of one species was affected at one organ site.

2,4-DMA therefore represents a major departure from the predictable pattern of aniline and o-toluidine. The site of tissue toxicity is shifted from the erythrocyte, spleen, and bone marrow to the liver. Significant species variation in susceptibility to its toxic effects, as well as type of organ pathology, is produced by 2,4-DMA in contrast to aniline and o-toluidine where these effects are relatively constant. 2,4-DMA, with metabolic activation, appears to be
positive in Salmonella mutagenicity tests whereas aniline and o-toluidine are negative. Limited carcinogenicity testing has failed to reveal any conclusive results regarding 2,4-DMA; yet aniline and o-toluidine have been shown to be carcinogenic. Finally, the metabolic products of 2,4-DMA are very different from those of aniline and o-toluidine, primarily due to the presence of a methyl group in the number 4 position.

2,6-Dimethylaniline

![Formula of 2,6-Dimethylaniline](image)

Figure 10. 2,6-Dimethylaniline.

2,6-Dimethylaniline (2,6-DMA, 2,6-xylidine, 2,6-dimethylbenzeneamine) is used as a chemical intermediate in the production of cosmetic and textile dyes. It is also a component of tobacco smoke and has been isolated as a metabolite of certain drugs and dyes.\(^{43}\) 2,6-DMA has been identified as a urinary metabolite in rats, guinea pigs, dogs, and man after administration of lidocaine, a local anesthetic.\(^{53}\) It is also a metabolite excreted in human urine after exposure to another xylidide local anesthetic, etidocaine, and is the major degradation product of lidamidine, an antidiarrheal agent.\(^{54,55}\) In cattle, an acid labile conjugate of 2,6-DMA is the major urinary metabolite of the sedative-analgesic, xylazine.\(^{56}\) The local anesthetics, mepivacaine and bupivacaine, both contain 2,6-DMA as part of their structures.\(^{57}\) It is
possible that these drugs are also metabolized to 2,6-DMA, at least in part.

Intestinal microflora were capable of cleaving azo dyes to aromatic amines.\textsuperscript{44} The dye Ponceau 3R yielded 1-amino-2-naphthol-3,6-disulfonic acid and several aniline derivatives on reductive splitting of the azo bond. Among the aniline derivatives, more than half were 2,4-, 2,5- and 2,6-dimethylanilines. 2,6-DMA accounted for 4-12\% of the metabolites of Ponceau 3R.

2,6-DMA may enter the environment from degradation of aniline based pesticides.\textsuperscript{58} \textsuperscript{14}C-labelled 2,6-DMA, when applied to soil, resulted in a binding of 66\% of the radioactivity after 24 hours. Acetylation apparently occurred readily in soil, and N-acetyl-2,6-DMA was isolated from soil treated with 2,6-DMA.

In a sub-acute study of the effects of several ring-substituted dialkylanilines, male Fischer 344 rats were gavaged with 2,6-DMA at 157.5 mg/kg/day (25\% of the LD\textsubscript{50}) for 5, 10 or 20 days.\textsuperscript{15} There was no change in splenic weight, but a significant increase in splenic hemosiderosis was seen. This increase was much smaller than that produced by aniline or o-toluidine, which were also included in the same study. 2,6-DMA produced no increase in splenic hematopoiesis, however, and bone marrow myeloid/erythroid ratios were normal. There were no microscopic lesions which could be ascribed to 2,6-DMA in the liver, thyroid, urinary bladder, kidney, trachea,
esophagus or bone marrow. These findings were in marked contrast to those in rats concurrently treated with 2,4-DMA. 2,4-DMA at a lower molar dose level (0.97 mmole/kg) than 2,6-DMA (1.30 mmole/kg) produced diffuse hepatocytic cloudy swelling and necrosis, periacinar necrosis with connective tissue deposition, periacinar vacuolar degeneration, and biliary hyperplasia. 2,6-DMA did not produce any significant lesions in the liver.

Aniline and o-toluidine were also included in the above study. The dimethyl anilines, 2,4- and 2,6-DMA, had a markedly reduced incidence and severity of lesions in the spleen and bone marrow. Thus, dimethyl substitution altered the toxicity of aniline to a substantial degree.

The lack of effect of 2,6-DMA in the rat was also demonstrated in an early study by Lindstrom. Rats were fed 2,4- or 2,6-DMA at 375, 750, 2500, 5000 or 10000 ppm for 6 months. There was a significant decrease in body weight at the two highest dose levels for both 2,4- and 2,6-DMA. There was also a significant increase in liver, kidney, spleen and testicular weights of males or females at the highest dose level. No microscopic hepatic lesions were observed for 2,6-DMA, whereas 2,4-DMA produced definite changes. Similar renal lesions for both isomers were seen in the 10000 ppm groups. Lindstrom concluded that the 2,4- isomer was the more toxic of the two and suggested that the difference in toxicity might be due to differences in metabolism.
Perhaps intrigued by Lindstrom's results, Magnusson et al. investigated the effects of 2,4-, 2,5- and 2,6-DMA in dogs and rats. Male and female beagles were treated orally with 2, 10 or 50 mg/kg/day of 2,4-, 2,5- or 2,6-DMA for 4 weeks. Intermittent vomiting was seen shortly after administration of all three compounds but occurred most often in 2,4-DMA treated dogs. Increased BSP retention was observed for all 3 isomers at the highest dose level and in a female dog given 10 mg/kg of 2,6-DMA. No effect was seen on blood glucose, blood urea nitrogen, hemoglobin, hematocrit, red and white blood cells or leucocyte differential count for any of the three isomers. Icterus was seen in dogs treated with 2,5- and 2,6-DMA. Fatty degeneration of the liver was seen with all doses of 2,6-DMA, at the middle and high dose levels of 2,5-DMA and at the high dose level of 2,4-DMA. There was a definite relationship between severity of fatty degeneration and dose of 2,6-DMA. Lipase-lead sulphide stains were positive for triglycerides in the liver.

In the same study, rats were treated with 20, 100 or 500-700 mg/kg/day of 2,4-, 2,5- or 2,6-DMA for 4 weeks. The high dose of all three isomers decreased erythrocytic hemoglobin concentration and, to a lesser extent, hematocrit. The livers of all treated rats were enlarged, and the enlargement was most severe and observed at all dose levels in rats given 2,4-DMA. Also, focal necrosis and vacuolization of hepatocytes were found in all rats given 2,4-DMA at the high dose. 2,6-DMA, at the same dose level,
produced focal necrosis in only 2 of 10 animals. The kidneys had a normal appearance in all rats. Magnusson concluded "that the toxicity of xylidines show great differences with regard to animal species and type of isomer". Based on doses required to produce toxicity, dogs appeared approximately 10 times more susceptible than rats to the toxic effects of the three isomers. Magnusson also agreed with Lindstrom's conclusion that the 2,4-isomer was more toxic to the rat than the 2,6-isomer. By using a second species, the dog, he was able to show that this relationship did not necessarily hold true for all species as the dog was more susceptible to the toxic effects of 2,6-DMA than the rat.

Magnusson performed a second study to further delineate the hepatic effects of the three isomers in the rat. Male and female Sprague-Dawley rats were gavaged once daily for 4 weeks with 400-500 mg/kg of 2,4-, 2,5- or 2,6-DMA. All three isomers caused hepatomegaly, with the greatest increase in weight occurring in the 2,4-DMA treated rats. A centrilobular decrease in liver glycogen was seen in all groups and was most evident in rats administered 2,4-DMA and least evident in rats treated with 2,6-DMA. In all three groups the decrease in glycogen was greater in males than in females. The same relationship was seen for hepatic glucose-6-phosphatase activity as for liver glycogen.

Electron microscopy showed proliferation of smooth endoplasmic reticulum in all treated rats; the proliferation was greatest in
2,4-DMA treated animals and least in rats dosed with 2,6-DMA. The hepatic microsomal protein content was increased in male and female rats treated with 2,4-DMA; a slight increase was seen in males dosed with 2,5-DMA. No effect on microsomal protein was seen with 2,6-DMA in males or females. Furthermore, 2,6-DMA did not increase hepatic microsomal cytochrome P-450, whereas both 2,4- and 2,5-DMA did increase hepatic cytochrome P-450 levels. Interestingly, glucuronyltransferase activity was increased in both sexes for all three isomers, and aniline hydroxylase was enhanced in all animals except males treated with 2,6-DMA. These results indicate that all three isomers, to varying degrees, can be inducers of drug metabolizing enzyme activity. 2,6-DMA appears to have less ability in this regard than the other two isomers.

It is important to note that the above studies on 2,6-, 2,4- or 2,5-DMA did not mention methemoglobin formation or its clinical consequence, cyanosis. Apparently, dimethyl substitution lowers the ability of aniline to form methemoglobin. Methemoglobin concentrations above 20% produce clinical signs of cyanosis in humans. 2,6-DMA given intravenously at 0.28 mmole/kg to cats, the most susceptible species to the methemoglobin forming effects of aromatic amines, resulted in formation of 10% methemoglobin. The same dose produced no effect in dogs. 2,4-, 2,5- or 2,6-DMA given by injection into the femur at 20 mg/rat produced methemoglobin levels of only about 3%. In cats, at doses of 0.25 mmoles/animal, 2,6- and 2,4-DMA induced methemoglobin levels of
10% compared to 35% for 2,5-DMA, 40% for p-toluidine, 60% for m-toluidine, 70% for o-toluidine and 72% for aniline. Obviously, the position of the methyl group as well as dimethyl substitution is important in methemoglobin induction. It is interesting that dimethyl substitution in the 2 and 5 positions roughly halves the methemoglobin percentage of o- or m-toluidine.

A search of the literature reveals one study on the biotransformation of 2,6-DMA. In 1963 Lindstrom et al. investigated the metabolism of this compound in rats. Male Osborne-Mendel rats were treated orally with 200 mg/kg/day for approximately eight days. Urinary metabolites detected using paper chromatography were para-hydroxy-2,6-DMA and a carboxylic acid derivative. The authors felt that other probable metabolites included the N-acetylated derivative of para-hydroxy-2,6-DMA and sulfate or glucuronide conjugates of this compound.

Concurrently treated 2,5-DMA rats also produced the para-hydroxylated product, as well as two carboxylic acid derivatives (oxidation at either the 2 or 5 position). However, the carboxylic acid derivatives of both 2,6- and 2,5-DMA were produced in smaller quantities than the para-hydroxylated compound. (Figure 11.)

A contrast exists between the metabolism of 2,4-DMA and 2,5- or 2,6-DMA in rats. 2,4-DMA was excreted primarily as a carboxylic acid derivative; whereas the other two isomers were mainly excreted as hydroxylated metabolites. Lindstrom felt that this difference may
Figure 11. Structures of identified and proposed metabolites of 2,6-DMA in the rat.
have been at least partially responsible for the difference in toxicity of the three isomers.

The absorption, distribution, elimination and bioretention of $^{14}$C-2,6-DMA has been investigated. Male and female Sprague-Dawley rats were gavaged with 63 mg/kg of the labeled compound. Rats receiving 10 daily doses of the labeled compound were compared to rats receiving a single dose after being pretreated with nine daily doses of the vehicle. Results indicated that a single oral dose of $^{14}$C-2,6-DMA was readily absorbed and distributed to rat organs and tissues. Tissues examined for radioactivity included whole blood, plasma, erythrocytes, liver, kidney, lung, brain, heart, spleen, pancreas, testes, epididymis, adrenal, thyroid, bone marrow, bladder, muscle, ovary and uterus. The label was eliminated primarily in urine and only to a minor extent in feces and expired air. Only small amounts of the radiolabel were recovered in tissues 24 hours post dosing. There was, however, some accumulation of radioactivity in the blood and other tissues in rats receiving 10 daily doses. This accumulation did not appear to be caused by impaired elimination as male rats excreted the radiolabel more rapidly in urine and feces than vehicle-pretreated rats receiving only one dose of the labeled compound. The greatest concentrations of radioactivity following the tenth repeated dose were found in red blood cells and liver. High concentrations, relative to other tissues, were also found in kidneys and whole blood. In another
study, distribution to nasal tissues of rats after a single dose of 63 mg/kg $^{14}$C-2,6-DMA was compared with distribution to other tissues. Twenty-four hours after gavage dosing, the concentration of radioactivity in nasal tissues was 2.5 times the concentration in liver. Concentration in the olfactory bulb was only slightly less than concentrations in the liver. These results were repeatable following intraperitoneal administration of the labeled compound as well.

The disposition of 2,6-DMA has been compared in two strains of rats. Male Fischer 344 and Sprague-Dawley rats were treated with a single oral dose of $^{14}$C-2,6-DMA (63 mg/kg). In Fischer rats, radioactivity in blood appeared and disappeared at faster rates, suggesting faster rates of absorption and elimination in this strain. However, there were no major differences in tissue concentrations at 24 hours after administration.

The National Toxicology Program (NTP) has conducted a carcinogenesis bioassay on 2,6-DMA. Male and female Charles River F1 CD rats were fed 2,6-DMA in the diet at 300, 1000 or 3000 ppm for 102 weeks. The F0 parents of these animals received the test diet at the same doses before breeding, during pregnancy and throughout the lactation period. Under the conditions of the bioassay, 2,6-DMA was carcinogenic in rats of each sex, causing significant increases in the incidences of both adenomas and carcinomas of the nasal cavity. A rare tumor of the nasal cavity, rhabdomyosarcoma, was observed in
dosed rats of both sexes (2 each in high dose males and females for a total of 4 in 112 animals). This type of tumor had not been previously reported at this site in Sprague-Dawley rats. Non-neoplastic nasal cavity lesions in high dose male and female rats included acute inflammation, epithelial hyperplasia and squamous metaplasia. Increased incidences of subcutaneous fibromas and fibrosarcomas in males and females and an increased incidence of neoplastic nodules in the livers of female rats were also observed.

Nasal cavity tumors have been associated with human occupational exposure and animal experimental exposure, primarily on inhalation. Occupations associated with an increased incidence of nasal tumors include the furniture and shoe industries and nickel refineries.\textsuperscript{64,65} Nasal cavity tumors in animals have been caused by formaldehyde,\textsuperscript{66} bis(chloro-methyl)ether,\textsuperscript{67,68} hexamethylphosphoramide,\textsuperscript{69} dichlorobutane,\textsuperscript{70} phenylglucidyl ether,\textsuperscript{71} dimethylcarbamoyl chloride,\textsuperscript{72} epichlorohydrin,\textsuperscript{73} 1,2-dibromo-3-chloropropane,\textsuperscript{74} and p-cresidine.\textsuperscript{75} The induction of nasal tumors by 2,6-DMA in the NTP bioassay is unusual as exposure was by the oral route. It may be argued that animals inhaled vapors given off by the dosed feed. However, oral gavage or intraperitoneal administration of labeled compound showed selective distribution to the nasal cavity.\textsuperscript{63} Inhalation exposure in these instances would have been minimal. The particular attributes of 2,6-DMA which caused this preferential distribution and tumor induction in the nasal cavity remain to be determined.
In conclusion, the toxic characteristics of 2,6-DMA are a radical departure from aniline, o-toluidine and its isomer, 2,4-DMA. The methemoglobinemia, splenic and bone marrow effects characteristic of aniline and o-toluidine are observed to only a minimal degree. In this respect, 2,6-DMA resembles 2,4-DMA. However, the toxicity, metabolism and carcinogenicity of the two dialkyl isomers appear to be quite different. 2,6-DMA induces hepatic lesions in the dog but only minimal changes in the rat, while 2,4-DMA displays the opposite species selectivity. In a limited study using simple techniques, 2,4-DMA has been reported to be primarily excreted as a carboxylic acid derivative and 2,6-DMA as the parahydroxylated product. The carcinogenicity of 2,4-DMA is equivocal, whereas 2,6-DMA induces both nasal cavity tumors and fibromas and fibrosarcomas of subcutaneous tissues.

Selected Methylene Bridged Anilines - MDA, MDPA, and MOCA

4,4'-methylene-bis-aniline (MDA)

4,4'-methylene-bis-diisopropyl-aniline (MDPA)

4,4'-methylene-bis-(2-chloro-aniline) (MOCA)

Figure 12. Structures of selected methylene bridged anilines.
Methylene bridged anilines were selected for review as examples of structurally complex aromatic amines for several reasons. Conjoined and condensed-ring aromatic amines (benzidine, 2-acetylaminofluorene) have been extensively studied and discussed. The methylene bridged anilines, although being used increasingly as replacements for conjoined anilines, have received comparatively little attention. These compounds are logical progressions in structural complexity from the single-ring aromatic amines and have an interesting set of toxicities.

4,4'-Methylene-bis-dianiline (MDA) and 4,4'-methylene-bis-(2-chloroaniline) (MOCA) are used in the plastics industry as curing agents and epoxy resin hardeners. 4,4'-Methylene-bis-diisopropyl-aniline (MDPA) is under development for the same purpose. Millions of pounds of MDA are produced in the United States annually, and although MOCA is no longer manufactured here, US companies imported 4 million pounds in 1980. MDA was considered responsible for an outbreak of toxic hepatitis in industrial workers. Exposure was thought to be primarily through skin absorption, but the possibility of inhalation or ingestion could not be ruled out. Symptoms included jaundice, abdominal pain, dark urine, pruritis and skin rashes, and elevated serum bilirubin and liver enzyme concentrations. One additional case of industrial exposure resulted in myocardial involvement (electrocardiogram abnormalities and LDH isoenzyme elevations) as well as toxic hepatitis. Non-industrial exposure and intoxication has also occurred; 84 persons unknowingly
ingested bread made from MDA-contaminated flour in Epping, England, and experienced hepatotoxicity. Liver biopsies showed cholangitis, cholestasis, biliary hyperplasia and portal inflammation with eosinophilic infiltration. Hepatocellular vacuolar degeneration was also described, but was a less prominent feature than the effects on the biliary system. Injury was reversible as all biopsies were normal several weeks after exposure. Liver damage has also been reported after acute or subchronic MDA administration in rats, rabbits, hamsters, dogs and cats. MDPA has also produced hepatotoxicity in rats and hamsters.

Short et al. gavaged hamsters for 5, 10 or 28 days with MDA at 87.5 mg/kg/day. Body weight was decreased on day 5, but weights were not significantly different from controls at days 10 and 28. There was no difference in liver weights of control and treated animals at any time. The predominant lesion in the liver, the only organ examined histologically, was a pronounced biliary hyperplasia and granulomatous cholangiohepatitis which progressed to biliary hyperplasia at 28 days.

Wistar rats were fed 1000 ppm MDA in the diet for 8, 16, 24, 32 or 40 weeks. Serial sacrifices were performed at each time period and animals from each group were sacrificed at the end of 40 weeks to evaluate recovery post dosing. Proliferation of bile ducts and oval cell formation, characterized by ovoid nuclei and small cellular-size, was seen in conjunction with replacement of hepatic parenchyma by
proliferating bile ducts. Development of portal cirrhosis was observed. However, these changes gradually reversed when the compound was withdrawn. Increases in serum glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase and gamma glutamic transpeptidase were found.

MDA, both with and without phenobarbitol-induced S-9 liver fraction, increased the number of revertant colonies in Salmonella typhimurium strains TA 100, TA 98, and TA 1538. A definite dose-response relationship with strain TA 100 was observed and MDA was judged mutagenic.

MDA was found to be carcinogenic in a bioassay performed by the National Toxicology Program (NTP). The hydrochloride salt of MDA was administered in the drinking water at 150 or 300 ppm for 103 weeks to male and female Fischer 344 rats and B6C3F1 mice. The incidence of thyroid neoplasms (carcinomas and adenomas) in the high-dose groups were elevated compared to control groups for both sexes of both species. Neoplastic hepatic nodules were observed in male rats, and hepatocellular tumors were found in male and female rats. Using a limited number of dogs, feeding low levels of MDA for 4-7 years did not produce evidence of carcinogenicity. Hepatotoxicity was observed, however, in the form of portal fibrosis, necrosis, and hemosiderosis. Interestingly, MDA administered in the 'post-induction' phase of N-ethyl-N-hydroxyethyl-nitrosamine treated rats inhibited liver, kidney and bladder carcinogenesis.
mechanism of this inhibitory effect is unknown, but may have been related to the decreased food consumption exhibited by MDA-treated rats or the toxic effects observed in the liver. Also, rats in this study developed goiter, providing a second report of a toxic effect of MDA on the thyroid. A goitrogenic effect was also seen in rats and mice fed another aniline derivative, 4,4'-oxydianiline.88

The metabolism of MDA has been studied in rats and rabbits.89 Rats excreted 91% of the administered dose (30 mg/kg) in 4 days with 56% recovered from the feces, while rabbits excreted 97% of which 16% was recovered from the feces. This is markedly different from aniline, o-toluidine, 2,4-DMA and 2,6-DMA which are primarily excreted in the urine. Both rats and rabbits excreted approximately 60% of the dose as unconjugated metabolites. This again is different from the metabolism of aniline and o-toluidine which are excreted mainly as conjugates. MDA (45%), N-acetyl MDA (11%), and N,N'-diacetyl-4,4'-diaminobenzhydrol (6%) were the major metabolites isolated from rabbit urine, with small quantities of azoxy, benzophenone and glycolamide metabolites also being identified. The diacetylated benzhydrol (42%) was the main metabolite in rat urine. Small amounts of acetylated phenols, benzophenones, and other MDA derivatives were also recovered. (Figure 13.)

Less information is available in the open literature concerning MDPA. In a study on the subacute toxicity of several ring-substituted dialkylanilines, MDPA was administered by gavage to male Fischer 344
Figure 13. Structures of the major urinary metabolites of MDA in rats and rabbits.
rats at 87.5 mg/kg (25% of the LD₅₀) for 5, 10 or 20 days. Body weights were significantly decreased on day 10, but not on days 5 and 20. Liver weights were significantly increased on all days. Liver lesions were very similar to those induced by 2,4-DMA (included in the same study), and were composed of diffuse, hepatocytic cloudy swelling and necrosis, periacinar necrosis with early connective tissue deposition, periacinar vacuolar degeneration, diffuse vacuolar change and biliary hyperplasia. There were no histopathologic compound-related effects in the kidneys, esophagus, trachea, thyroid, parathyroid or urinary bladder.

In a later study, Short et al. evaluated the effect of dose on MDPA toxicity in the rat and hamster. Male Fischer 344 rats were administered the compound by gavage at 10.5, 21.0, 42.0, 63.0, or 87.5 mg/kg for 5, 10 or 28 days. Hamsters received 87.5 or 875 mg MDPA/kg daily for the same periods. Histopathologic examination revealed diffuse vacuolar change and periacinar vacuolar degeneration in the livers and congestion, hemosiderosis and hematopoiesis in the spleens of rats. Even in the face of continued dosing, hepatic vacuolar degeneration decreased in incidence and severity from day 5 to day 28. Livers of rats sacrificed 28 days after cessation of MDPA treatment had recovered and were normal on histopathologic examination. At 87.5 mg/kg (equal to the high dose in rats), the only lesion observed in hamsters was periacinar vacuolar change. Ten times this dose produced hepatic periacinar vacuolar change, vacuolar degeneration, hepatocytic swelling and necrosis, toxic tubular
nephrosis and a high mortality rate (90% in less than 10 days). This high mortality rate was unexpected as a dose-range finding study using single doses of MDPA as high as 5000 mg/kg failed to produce greater than 25% mortality.

Blood glucose, blood urea nitrogen (BUN), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and red blood cell Heinz body formation were also evaluated in rats in the above study.76 There were no significant differences between controls and dosed animals for BUN, ALT, and AST. No Heinz bodies were found on blood smears. Significant differences, however, were found in blood glucose concentrations. Blood glucose levels were reduced on day 5 in the 3 highest dose levels and in the two highest dose levels on days 10 and 28. Values had returned to normal 28 days after cessation of treatment. Decreased food intake was not felt to be responsible for the decrease in blood glucose levels as there was no evidence of starvation. No difference between control and treated animals for body weight or urinary ketone levels was observed.

Mitochondrial function was altered by MDPA treatment.76 Stage III, Stage IV, and maximal respiratory rates of mitochondria isolated from rats treated for 28 days with 87.5 mg/kg were higher than age matched controls.

Perhaps because of its extensive use in the plastics industry and the potential for human exposure, much of the literature concerning the
toxic effects of MOCA centers on its potential for carcinogenicity. Human exposure has occurred during its manufacture and use, and persons living in the area of its manufacture have absorbed MOCA as evidenced by the presence of parent compound in their urine. Thus far, no epidemiological evidence exists which implicates the induction of bladder cancer as an occupational hazard of MOCA. However, six male dogs were given MOCA for up to 9 years at 100 mg/day for 5 days/week per os; five of the dogs surviving more than 8 years had transitional cell carcinomas of the urinary bladder. It has also been demonstrated to be carcinogenic in the mouse and rat after oral administration, and to produce tumors distant from the site of injection by subcutaneous administration. Lung tumors in both mice and rats were the most striking observation. Finally, MOCA is mutagenic in the Ames Salmonella assay and mouse lymphoma assay, and is genotoxic in isolated mouse and hamster hepatocytes.

MOCA was rapidly metabolized and excreted in rats and dogs. Following intravenous, intraperitoneal or per os administration of $^{14}$C-MOCA to rats, the highest concentrations of radioactivity were found in the small intestine, liver, adipose tissue, kidney, and lung. In dogs given $^{14}$C-MOCA intravenously, the highest concentrations were found in bile, liver, kidney, fat and lung. A high proportion of the administered dose was excreted in the feces; in this respect MOCA was similar to MDA. Extensive metabolism of the parent compound took place and the results of several studies have
all reported only very small quantities of parent compound in the urine. Only one report on the metabolism of MOCA is available in the literature. Manis and Braselton characterized the major urinary metabolite (the only metabolite investigated) in dogs using HPLC, mass spectrometry, and nuclear magnetic response. The metabolite was identified as 5-hydroxy-3,3'-dichloro-4,4'-diaminodi-phenylmethane-5-sulfate. (Figure 14.)

Upon hydrolysis with arylsulfatase, the metabolite bound to both DNA and protein although it was unreactive as the sulfate conjugate. Lack of mutagenicity, upon hydrolysis, was observed with strain TA 1538 Salmonella typhimurium. Thus, it was concluded that the ortho-hydroxylated sulfate conjugate was unreactive, but that a more labile conjugate or the hydroxylated compound could be reactive and may contribute to the toxicity and carcinogenicity of MOCA.90

The ortho hydroxylation and sulfate conjugation of MOCA that occurred in the dog is in marked contrast to the pathway of MDA metabolism in the rat and rabbit. In these species metabolism proceeded primarily by oxidation of the methylene bridge and acetylation of the nitrogen atom. This difference in biotransformation may be the result of species variation in metabolism rather than differences in chemical
structure of the two compounds. Carnivores, as opposed to rodents and rabbits, appear to preferentially oxidize aniline in the ortho-rather than the para-position. With only a limited number of studies for comparison, this relationship appeared to hold true for MOCA metabolism in the dog (o-hydroxylation) and MDA metabolism in the rat and rabbit (hydroxylation of the methylene bridge para to the nitrogen). It is of interest to note that Manis and Braselton have been unable to isolate the ortho-hydroxysulfate conjugate of MOCA from the urine of exposed industrial workers. Perhaps in man, the metabolic pathway of MOCA resembles that of the rat and rabbit more closely than that of the dog.

In conclusion, upon acute or sub-chronic exposure MDA produced its major toxic effect on the hepatic biliary tree. The histopathologic lesions were markedly similar in the human, hamster, and rat. Only a single study has been performed to investigate the toxic effects of MDA in the dog, however, long-term feeding of this compound in this species produced portal fibrosis. This finding may well have been an extension of earlier biliary hyperplasia and thus it is possible that MDA hepatic lesions hold constant in this species as well. The histopathologic lesions induced by MDA were in marked contrast to those produced by the simpler aromatic amines aniline, o-toluidine, 2,4-DMA and 2,6-DMA. The toxic effects of aniline and o-toluidine were limited to the erythrocyte, spleen and bone marrow, and while 2,4- and 2,6-DMA were hepatotoxic, this toxicity exhibits species variation and does not resemble that of MDA. MDPA, however, induced
hepatocellular vacuolar degeneration in rats and hamsters and a limited degree of splenic hemosiderosis and hematopoiesis in rats, lesions which were similar to those induced by the dialkylaniline, 2,4-DMA. Species variation in susceptibility to the toxic effects of MDPA existed, as hamsters were much more resistant than the rat and required significantly higher doses to develop serious pathology. Furthermore, not only were hepatic lesions reversible following cessation of treatment in the rat, but liver damage declined in both incidence and severity in the face of continued dosing. With respect to carcinogenicity, MDA and MOCA have produced tumors in rats, mice, and/or dogs. MDA has induced hepatocellular and thyroid tumors in rats, but was non-carcinogenic in the dog. Bladder tumors were demonstrated in dogs following long-term MOCA administration, whereas lung tumors were induced in rats and mice. MDPA has not been tested in this regard. Of the simpler aromatic amines discussed in this review, only o-toluidine has produced tumors at any of the above sites (bladder, liver).
The primary goal of this study is to determine whether or not the species specific hepatic damage caused by 2,4-DMA and 2,6-DMA is related to different patterns of metabolism. As stated in the Historical Review, the rat and dog differ markedly in their susceptibility to the induction of toxic lesions in the liver. Furthermore, the types of lesions produced differed in the 2 species. 2,4-DMA has been shown to produce biliary hyperplasia in the rat, but produces virtually no lesion in the dog. In contrast, 2,6-DMA has been shown to produce fatty degeneration in the dog but it is virtually without effect in the rat. This study seeks to determine whether or not the difference can be explained by differences in the metabolism of 2,4-DMA and 2,6-DMA in these 2 species. It is probable, for example, that 2,4-DMA undergoes oxidative metabolism to a reactive metabolite (or intermediate) in the rat which is not produced in the dog. On the other hand 2,6-DMA may be metabolized to a toxic product in the dog but not in the rat. Since the only major lesions of these 2 chemicals are found in the liver, and since the hepatic mixed function oxidase system is the principle source of biotransformation reactions in the body, it is logical to suspect that these lesions might be the result of locally produced reactive metabolites.

The approach to testing this hypothesis will be to combine a
histopathologic investigation with a study of the hepatic metabolism of 2,4-DMA and 2,6-DMA in the dog and rat. The intent of this approach is to verify the production of the lesions and to see if a correlation exists between the appearance of specific metabolites and lesions. In the event that specific lesions are correlated with the production of certain metabolites, a tentative hypothesis regarding the generation of reactive metabolites (or intermediates) may be possible. Finally, it is also possible that reactive metabolites may bind covalently to hepatic subcellular fractions. This will be investigated by determining the covalent binding of the radiolabelled compounds to hepatocellular DNA, RNA and protein.

The hepatic effects of the compounds in each species will be investigated using light microscopy and special staining techniques for lipids, and by electron microscopy. Microscopic examination of liver specimens will be performed after 10 days of treatment with either 2,4- or 2,6-DMA in the dog and rat. In addition, rats will be pretreated with the drug metabolizing enzyme inducers, phenobarbital or 3-methylcholanthrene, and the enzyme inhibitor, proadifen (SKF-525A), to determine the effects of alterations in biotransformation on liver lesions. Phenobarbital (PB) induces the cytochrome P-450 containing mixed function oxidase, whereas 3-methylcholanthrene (3MC) is an inducer of cytochrome P-448. The specific pathways affected by these two inducers may differ, and thus pretreatment may substantially effect hepatic lesions. Either a decrease or an enhancement of the toxic effects may occur, depending on the balance between the induction of activation vs. detoxification pathways. SKF-525A, an inhibitor of the
mixed function oxidase system, should substantially reduce liver lesions if these lesions are the result of the formation of a toxic metabolite. Urinary metabolite studies will determine the identity and quantity of metabolites produced by the dog and rat. By comparing hepatic lesions with metabolite production in the two species and also between rats treated with phenobarbital, 3-methylcholanthrene, or SKF-525A, it may be possible to identify the toxic metabolite(s). An attempt to correlate liver lesions with species differences in metabolism, susceptibility, and dose will be made as well. Furthermore, collection of urine on days 1 and 10 of treatment with 2,4- or 2,6-DMA will determine if metabolite formation differs in the naive and subchronically exposed animal.
MATERIALS AND METHODS

Chemicals

The dimethylanilines (2,4- and 2,6-DMA), both of 99% purity, were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. The 2,6-DMA \([\text{Ring-}^{14}\text{C(u)}]*\text{HCl}\) (10.90 mCi/m mole, >99% purity) and 2,4-DMA \([\text{Ring-}^{14}\text{C(u)}]*\text{HCl}\) (20.3 mCi/m mole, >99% purity) were custom synthesized by New England Nuclear, Boston, MA. Metabolite standards N-hydroxy-2, 6-DMA, N-acetyl-2,6-DMA, 6-hydroxy-2,4-DMA as the N-sulfate salt, 2,6-dimethyl-nitrosobenzene, 4-hydroxy-2,6-dimethyl-nitrobenzene, 4-N-hydroxy-imino-3,5-dimethyl-quinone, N-acetyl-2,4-DMA, and 2,4-dimethyl-nitrosobenzene were synthesized by George Anderson, Ph.D., Baton Rouge, LA. Structures were confirmed by Dr. Anderson on the basis of melting point and nuclear magnetic resonance analysis. Standards of the metabolites N,2,4-trimethylaniline, N,2,5-trimethylaniline, N-acetyl-4-amino-3-methyl-benzoic acid, 2-amino-3-methylbenzyl alcohol and 2-amino-3-methyl-benzaldehyde were synthesized by Steven A. Baker, Ph.D., LSU School of Veterinary Medicine, Baton Rouge, LA. The chemical standard of 4-hydroxy-2,6-dimethylaniline was isolated from the urine of rats treated with 2,6-DMA. Purification of the metabolite was performed by Dr. Barker. Structures of above metabolites were confirmed by mass spectrometry and/or nuclear magnetic resonance.
2-Amino-5-methyl-benzoic acid, 4-amino-3-methyl-benzoic acid, and 2-amino-3-methyl-benzoic acid were purchased from Aldrich Chemical. o-Toluidine (98% purity) was obtained from Matheson, Coleman, and Bell Manufacturing Chemists, Norwood, OH. 3-Methylcholanthrene (3MC) and phenobarbital (PB, Luminol®) were purchased from Eastman Kodak, Rochester, NY and Winthrop Laboratories, New York, NY, respectively.

SKF-525A was a generous gift of Smith, Klein, and French Laboratories, Philadelphia, PA. All other chemicals were of reagent grade or better and obtained from commercial sources.

Animals

Twelve-week-old male Fischer 344 rats were obtained from Hilltop Lab Animals, Inc., Scottdale, PA, and conditioned for 2 weeks. Food (Purina Rat Chow) and water were allowed ad libitum. Animals were housed 2 or 4 per cage. A 12/12 hr light/dark cycle was maintained throughout the conditioning and treatment periods.

Ten male purebred Marshall Beagles (Louisiana State University School of Veterinary Medicine breeding program), aged 2 years, were housed in individual cages, fed Purina Field and Farm Dog Chow, and allowed water ad libitum. A 12/12 hr light/dark cycle was maintained.
Experimental Design

This study was designed to investigate the urinary metabolite profile (rat, dog), hepatotoxicity (rat, dog), and covalent binding (rat) of 2,4- and 2,6-DMA. Urinary metabolites of both compounds in both species were isolated following 1 or 10 consecutive oral doses. Liver samples (by biopsy or at sacrifice) were taken following the tenth day of treatment. In addition, the effects on urinary metabolites and hepatic lesions of 3 compounds which alter xenobiotic metabolism (PB, 3MC, SKF-525A) were investigated in the rat, as was the covalent binding of the two test compounds to liver DNA, RNA, and protein.

Hepatotoxicity and Urinary Metabolite Identification in the Rat

The rat hepatotoxicity and in vivo metabolism segments of the study were initiated with 16 animals allocated to each of 12 groups; 8 test groups and 4 groups of controls. Test groups 1-4 received 117 mg/kg/day of 2,4-DMA in a corn oil gavage for 10 days; groups 5-8 received 2,6-DMA at 262.5 mg/kg/day in the same manner. The dose of the test substances was equal to 25% of their respective LD50 values (LD50, oral 2,4-DMA = 467 mg/kg; revised LD50, oral 2,6-DMA = 1050 mg/kg\textsuperscript{15}). In addition to the test substance, groups 2-4 received PB (80 mg/kg/day), 3MC (15 mg/kg/day in corn oil), or SKF-525A (50 mg/kg/day in sterile water), respectively, via intraperitoneal (IP) administration once every twenty-four hours for 10 days. Groups 6-8 were treated in a manner identical to that of groups 2-4 with respect to enzyme induction or inhibition. An age-matched set of controls was used for each treatment group. The
control animals were gavaged with corn oil and treated with the appropriate enzyme inducer/inhibitor (Groups 9-12). Animals were weighed every 5 days and doses were adjusted accordingly (Table 1).

Pooled 24 hour urine samples were collected on Days 1 and 10 for all treatment groups and on Day 5 for SKF-525A treated animals. Rats within the same treatment group were housed 2 per stainless steel metabolism cage, resulting in a total of 8 urine samples/group. Immediately after collection, urine was filtered (Whatman #4 filter paper) and stored at -10°C. Urine samples were later analyzed for parent compounds and metabolites.

Following 10 consecutive daily doses of the test substance, animals were sacrificed on Day 11 by induction of anoxic narcosis in an atmosphere of CO₂ followed by exsanguination. Total liver weight was recorded and 3 samples/liver were preserved for light and electron microscopy. Samples preserved for histopathologic evaluation were fixed in 10% neutral buffered formalin; samples retained for electron microscopic examination were fixed in 1.25% gluteraldehyde/2% formalin in 0.1 M sodium cacodylate buffer. A final sample was quick frozen in a dry ice/acetone bath and stored at -10°C for future evaluation of lipid content (oil red O stain).

Unexpected mortality in the PB:2,4-DMA (Group 2) rats required an alteration in the above protocol (Table 1). Twenty four hours following the fifth consecutive dose, all remaining animals in the PB:2,4-DMA group (Group 2, n = 8) were sacrificed and tissues obtained for
Table 1. Description of Groups and Treatments for Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Test Compound</th>
<th>Dose (mg/kg/day)</th>
<th>Corn Oil Gavage</th>
<th>Enzymatic Modifiers (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,4-DMA</td>
<td>117*</td>
<td>vehicle</td>
<td>PB 3MC SKF</td>
</tr>
<tr>
<td>2</td>
<td>2,4-DMA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>80+</td>
</tr>
<tr>
<td>3</td>
<td>2,4-DMA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>15+++</td>
</tr>
<tr>
<td>4</td>
<td>2,4-DMA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>- 50++</td>
</tr>
<tr>
<td>5</td>
<td>2,6-DMA</td>
<td>262.5*</td>
<td>vehicle</td>
<td>PB 3MC SKF</td>
</tr>
<tr>
<td>6</td>
<td>2,6-DMA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>80 -</td>
</tr>
<tr>
<td>7</td>
<td>2,6-DMA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>- 15 -</td>
</tr>
<tr>
<td>8</td>
<td>2,6-DMA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>- 50</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>-</td>
<td>0.2ml/250g Bwt</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>PB-Control</td>
<td>-</td>
<td>&quot;</td>
<td>80 -</td>
</tr>
<tr>
<td>11</td>
<td>3MC-Control</td>
<td>-</td>
<td>&quot;</td>
<td>- 15 -</td>
</tr>
<tr>
<td>12</td>
<td>SKF-Control</td>
<td>-</td>
<td>&quot;</td>
<td>- 50</td>
</tr>
</tbody>
</table>

* administered as a corn oil gavage; 0.2 ml/250g Bwt
+ administered via IP injection as phenobarbital sodium
++ administered via IP injection in corn oil; 0.15ml/250g Bwt
+++ administered via IP injection in sterile water 1 hr prior to oral gavage with test compound; 0.15 ml/250g Bwt
histopathologic and electron microscopic evaluation as described. A corresponding number of PB:Control (Group 10a) and PB:2,6-DMA (Group 6a) animals were also sacrificed at this time for the sake of comparison. The remaining animals in the above 2 groups (n = 8/group) were treated and sacrificed according to the original protocol (Table 2). Urine samples were collected for 24 hours from all animals sacrificed on Day 6, and were then filtered, and frozen. All rats sacrificed on Day 6, as well as the remaining PB:Controls (Group 10b) and PB:2,6-DMA (Group 6b) rats sacrificed on Day 11, were housed individually in metabolism cages.

Hepatotoxicity And Urinary Metabolite Identification

In the Dog

The dog hepatotoxicity segment of the study was initiated with 2 groups of 5 animals each. Group 1 received 2,4-DMA at 25 mg/kg/day and group 2 received 2,6-DMA at the same dose for a period of 10 days. Prior to the administration of the test substance, all 10 dogs underwent surgery for collection of control liver samples for light and electron microscopic evaluation. One dog from each group underwent surgery each day. A total of 2 dogs/day were subjected to the surgical procedure.

Food and water were withheld 24 hours preceding surgery. Anesthesia was induced with thiamylal sodium (Bio-Tal®, Bio Ceutic, St. Joseph, MO; 22 mg/kg IV) and maintained with halothane (Halothane U.S.P., Halocarbon Laboratories, Inc., Hackensack, NJ; 1% flow rate). Using a ventral midline incision a portion of the left lateral lobe of the liver was isolated and a venal caval clamp applied crosswise approximately 3 cm
Table 2. Number of Animals per Group and Day of Sacrifice

<table>
<thead>
<tr>
<th>Group</th>
<th>Test Compound</th>
<th>Enzymatic Modifiers</th>
<th>n</th>
<th>Day of Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,4-DMA</td>
<td>None</td>
<td>16</td>
<td>11*</td>
</tr>
<tr>
<td>2</td>
<td>2,4-DMA</td>
<td>PB</td>
<td>8</td>
<td>6**</td>
</tr>
<tr>
<td>3</td>
<td>2,4-DMA</td>
<td>3MC</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>2,4-DMA</td>
<td>SKF-525A</td>
<td>15+</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>2,6-DMA</td>
<td>None</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>6a</td>
<td>2,6-DMA</td>
<td>PB</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>6b</td>
<td>2,6-DMA</td>
<td>PB</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>2,6-DMA</td>
<td>3MC</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>2,6-DMA</td>
<td>SKF-525A</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>None</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>10a</td>
<td>PB-Control</td>
<td>PB</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>10b</td>
<td>PB-Control</td>
<td>PB</td>
<td>7+</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>3MC-Control</td>
<td>3MC</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>12</td>
<td>SKF-Control</td>
<td>SKF-525A</td>
<td>15+</td>
<td>11</td>
</tr>
</tbody>
</table>

* Sacrificed 24 hours after the 10th consecutive day of daily dosing.
** Sacrificed 24 hours after the 5th consecutive day of daily dosing.
+ One of the original 16 animals died before the scheduled date of sacrifice.
from the lobe's apex. The isolated liver wedge was removed with scissors distal to the clamp and processed immediately. The defect was oversewn by preplacing sutures prior to removal of the clamp. Using this technique, hemorrhage from the resected liver was minimal. The abdomen was closed in a standard manner consistent with good surgical technique. Preservation of liver samples for histopathology, electron microscopy and lipid staining was conducted as described for the rat.

A rest period of 21 days was allowed between the initial surgeries and the administration of 2,4- or 2,6-DMA. The test substance was administered orally in gelatin capsules with no vehicle. Dogs were weighed every 5 days and doses were adjusted in order to maintain a constant weight/weight (mg/kg) dose. On Days 1 and 10 of treatment, 24 hour urine samples were collected after placing the animals in stainless steel metabolism cages. Urine was filtered under vacuum (Whatman #4 filter paper) immediately after collection and stored at -10°C.

A liver biopsy was collected from each dog on Day 11 of treatment. One dog from each treatment group underwent surgery each day, and a total of two surgeries/day were performed. Food and water were withheld for 24 hours preceding surgery. Atropine sulfate (0.07 mg/kg) (Atropine Injectable S.A., Fort Dodge Laboratories, Fort Dodge, IA) was used as a preanesthetic. Induction and maintenance of anesthesia were achieved with thiamylal sodium (22 mg/kg) and halothane, (1%) respectively. A ventral midline incision was made in order to expose the liver and a disposable biopsy needle (Tru-Cut® 15.2 cm cannula, 20 mm specimen notch, Travenol Laboratories, Deerfield, IL) was inserted through the
incision into the liver. The abdominal incision was closed in a standard manner. No deaths occurred during the treatment or post-operative periods.

Liver tissue removed via the biopsy was processed immediately after collection in the same manner as that described for the rat.

Covalent Binding to Hepatic DNA, RNA, and Protein of the Rat

The covalent binding of $^{14}C\text{-}2,4\text{-DMA}$ and $^{14}C\text{-}2,6\text{-DMA}$ to hepatic DNA, RNA, or protein was investigated using 2 test groups of 24 rats each. Hepatic DNA, RNA and protein were isolated from an age-matched non-treated control group ($n = 12$) in order to determine background radioactivity in the untreated rat. Animals were randomly assigned to 2 of the 3 groups. Each group was further subdivided into blocks of 4 rats each (treatment groups: 6 blocks of 4 animals each, control groups: 3 blocks of 4 animals each). 2,4- or 2,6-DMA was administered to the test groups by oral gavage at 117 mg/kg/day and 262.5 mg/kg/day, respectively, in a corn oil vehicle for 9 days. On Day 10 of treatment, $^{14}C\text{-ring-labelled-2,4\text{-DMA (HCl salt)}}$ (0.0872 mCi/rat, 0.008 mmoles/rat, dissolved in ethanol) or 2,6-DMA (HCl salt) (0.0872 mCi/rat, 0.008 mmoles/rat, dissolved in sterile water) was administered by IP injection. Rats were weighed every 5 days and doses recalculated. Control animals received no treatment whatsoever, i.e., they were not gavaged.
Twenty four hours post dosing with radiolabelled compound, animals were sacrificed by CO₂ exposure and exsanguination. Total liver weight was recorded and a small wedge removed and weighed. Liver wedges from 4 rats constituting a block were pooled (approximately 3 g/block) and stored at -10°C. All DNA, RNA, protein and covalent binding determinations were made on the basis of the pooled tissues of 4 rats.

**Histopathologic and Electron Microscopic Evaluation**

For histopathologic evaluation, formalin fixed liver samples were embedded in paraffin, sectioned and stained with hematoxalin and eosin. Selected liver samples were also evaluated 1) utilizing frozen sections stained with oil red O for confirmation of lipid inclusions and 2) via transmission electron microscopy using epon embedded sections stained with uranyl acetate and lead citrate.

**Metabolite Isolation and Identification**

**Preliminary Study**

The goals of the preliminary study were two-fold. The first goal was to develop suitable extraction and chromatography methods for the analysis of metabolite standards. These methods were not available in the literature. The second goal was to identify the major and minor metabolites present in the urine of dogs and rats treated with 2,4- or 2,6-DMA prior to quantification. Previous studies utilized paper
chromatography to isolate and identify metabolites in urine.\textsuperscript{44,45} A thorough investigation utilizing more definitive techniques was needed.

The preliminary study was conducted using blank urine spiked with metabolite standards or the pooled 24 hour urine samples of 4 rats or dogs. Urine from days 1, 5 and 10 of treatment was thawed and mixed on a vortex shaker. Three 2 ml aliquots per pooled urine sample were utilized for analysis. Aliquots were subjected to treatment with either \(\beta\)-glucuronidase (Type L-II, Sigma Chemical Co., St. Louis, MO) or arylsulfatase (Type H-I, Sigma Chemical Co., St. Louis, MO) to liberate parent and metabolites conjugated with glucuronic acid or sulfate, respectively. A third aliquot was extracted without prior hydrolysis for quantification of free (non-conjugated) parent and metabolites.

Enzymatic hydrolysis proceeded as follows. For determination of glucuronide conjugates, a 2 ml urine aliquot was incubated at 45°C for 1 hr in a shaking water bath with 2000 U \(\beta\)-glucuronidase and 2 ml 0.2 M \(\text{KH}_2\text{PO}_4\) buffer, pH 3.8. These conditions inhibit the contaminating sulfatase activity in the enzyme preparation.\textsuperscript{102} Sulfate conjugates were liberated under the same incubation conditions utilizing 2 ml of urine, 20 mg arylsulfatase, and 2 ml Na acetate buffer, pH 5 (0.2 Na acetate, 0.2 M ascorbic acid, 0.2 M D-saccharic acid-1,4-lactone). Na acetate buffer containing D-saccharic-acid-1,4-lactone was added to inhibit any contaminating glucuronidase activity\textsuperscript{103}. An internal standard (o-toluidine) was added prior to incubation.
Studies conducted using spiked urine samples indicated that all 2,6-DMA and 2,4-DMA standards, excepting 6-hydroxy-2,4-DMA, were adequately extracted with ethyl acetate from aqueous solutions at a pH of 3. 6-Hydroxy-2,4-DMA was extractable at a pH of 7.4. Therefore, following enzymatic hydrolysis of 2,6-DMA samples, the pH was adjusted to 7.4 with NaOH and 2 ml ethyl acetate were added. The mixture was vortexed briefly and centrifuged for 10 minutes at 2500 rpm on a clinical centrifuge (Sorvall GLC-28 General Laboratory Centrifuge, Du Pont Instruments, Newtown, CT). The organic layer was removed and the extraction process repeated twice more and pooled. The aqueous layer was then adjusted to pH 3 with HCl and extracted 3 times with ethyl acetate as described. The organic layers from each pH extraction were combined and evaporated to dryness under a N₂ stream at room temperature. In order to achieve adequate chromatography of carboxylic acid metabolites, the extract was derivatized by incubating for 1 hour with 200 µl of an ether solution containing diazomethane. The diazomethane solution was prepared immediately before use by adding 20 mg of N-nitroso-N-methylurea (Sigma Chemical Co., St. Louis, MO) to 1 ml of 45% KOH and 2 ml ethyl ether while stirring. After incubation, the solution was evaporated to dryness under N₂ and dissolved in 0.5 ml ethyl acetate for gas chromatography/mass spectrometry (GC/MS) analysis. Gas chromatographic samples were diluted by adding 100 µl of the above solution to 400 µl of ethyl acetate.

Extraction of the non-hydrolyzed 2,6-DMA urine samples proceeded in the same manner except that 1 ml of ethyl acetate used for each of three separate extractions.
Urine from animals treated with 2,4-DMA was extracted in the same manner as described above for 2,6-DMA samples.

Retention times of metabolites and normal urinary constituents were identified utilizing a Varian 6000 Gas Chromatograph equipped with a Varian 4290 Integrator and a 30 meter 0.32 mm ID, 0.2 mm coated DBS FSOT capillary column. Injections were made in the splitless mode with a septum purge (60 ml/min) being initiated 0.5 min post injection. The injection port and detector (nitrogen-phosphorus specific) temperatures were 200°C and 300°C, respectively. Helium (1 ml/min, 15 PSI head pressure) was the carrier gas. For samples containing 2,6-DMA and its metabolites, the following temperature program was used: 50°C initial temperature, 1 min hold, 5°C/min ramp to 120°C, 5 min hold, 10°C ramp to 220°C, 10 min hold. Samples containing 2,4-DMA and its metabolites were analyzed using a temperature program of 50°C initial temperature, 1 min hold, 10°C ramp to 220°C, 22 min hold.

Mass spectra were obtained with a Finnigan TSQ45A triple quadrupole mass spectrometer with gas chromatograph (GC/MS/MS/DS) operating in the electron impact, positive ion mode. The emission current was set at 0.3 amps with an electron energy of 70 eV. The instrument was tuned daily with perfluorotributylamine. Transfer zone temperatures were 300°C. Gas chromatographic conditions were as follows: the injection port temperature equalled 350°C; oven temperature was at 50°C for 1 min followed by a 10°C/min ramp to 300°C, followed by a 10 min hold; He carrier gas flow at 1 ml/min (15 PSI head pressure); purge function (60
ml/min) initiated at 0.5 min post injection. A 30 m, 0.2 mm ID, Hewlett Packard SE 54 (0.1 mm coating) column operating in the splitless mode was used for chromatographic separations.

Metabolite Quantitation

The extraction procedure for the quantitation of metabolite levels was altered based on the results of the preliminary study. Smaller quantities of urine were extracted to achieve final concentrations of parent compounds and major metabolites suitable for quantitation. Evaporation under a nitrogen stream was deleted to avoid losses of volatile compounds and spurious elevations in concentrations of non-volatile metabolites. GC and GC/MS parameters were also altered to decrease analysis time.

Frozen urine samples, each representing the 24 hour urine production of two rats, were thawed and mixed on a vortex shaker. The urine samples were paired, and an aliquot of 200 μl was withdrawn from each sample and pooled, producing a volume of 400 μl. Each pooled aliquot represented the urine of 4 rats. (For Groups 2, 6a, and 6b each 400 μl aliquot represented the pooled urine of 2 rats.) Three 400 μl aliquots from each pair were extracted without prior enzymatic hydrolysis, after incubation with sulfatase or after incubation with glucuronidase. Thus, 12 urine aliquots were extracted and analysed per treatment group (4 urine aliquots/treatment group each undergoing no hydrolysis, sulfatase hydrolysis and glucuronidase hydrolysis).
After pooling and following the addition of 1.5 ml of water, the urine from rats treated with 2,4-DMA was adjusted to pH 3 with 1 N HCl. o-Toluidine, as internal standard, was added [200 μl, (1 ppt)], followed by 650 μl ethyl acetate. The mixture was vortexed and centrifuged as described in the preliminary study, and the organic layer removed. The extraction was repeated using ethyl acetate, and the organic layers combined and treated with diazomethane (500 μl) as previously described. No evaporation with N₂ was attempted, however.

For liberation of sulfate and glucuronide conjugates, two 200 μl aliquots were withdrawn from each urine sample, combined as described above, and subjected to enzymatic hydrolysis. Incubation ratios, times and temperatures were as described in the Preliminary Study. Extraction was identical to that of the nonhydrolysed sample with the exception that water was not added to hydrolysed aliquots; this volume being replaced by incubation buffer.

Pooling and extraction of urine from rats treated with 2,6-DMA was identical to that described above except that the extraction was carried out at pH 7.4-8.4 and no derivatization was performed.

Aliquots of urine from dogs treated with 2,4- or 2,6-DMA were extracted and analysed individually. Urine samples were thawed, vortexed, and a 1 ml aliquot added to 1 ml of water. The pH was adjusted to 7.4-8.4 with 0.1 N HCl, o-toluidine added as internal standard (200 μl, 1ppt) and the mixture extracted twice with ethyl acetate (650 μl). The pH of the aqueous phase was then adjusted to 3 (0.1 N HCl) and extracted once with
650 µl of ethyl acetate. The organic layers were combined and treated with diazomethane as previously described.

Enzymatic hydrolysis of dog urine samples was performed using the same methods described for rat urine. A urine volume of 1 ml, however, was subjected to enzymatic hydrolysis.

Metabolites were quantified utilizing a Varian 6000 Gas Chromatograph equipped with a Varian 8000 Autosampler and Vista 402 Data System. A 30 meter 0.32 mm ID, 0.2 coated DB5 FSOT capillary column operated in the splitless mode was used. Septum purge (60 ml/min) was initiated 0.5 min post injection. The injection port and detector (nitrogen – phosphorus specific) temperatures were 200° and 300° C, respectively. Helium (1 ml/min, 15 PSI head pressure) was the carrier gas. Urinary extracts from animals treated with 2,4-DMA and from rats treated with 2,6-DMA were analysed using the following temperature program: 60°C initial temperature, 1 min hold, 20°C/min ramp to 220°C, 10 min hold [Temperature Program 1 (TP-1)]. Urinary extracts from dogs treated with 2,6-DMA were analyzed using a temperature program of 60°C initial temperature, 3 min hold, 10°C/min ramp to 220°C, 10 min hold [Temperature Program 2 (TP-2)].

Mass spectra were obtained with the same instrument and conditions as described in the Preliminary Study. Gas chromatographic conditions, however, were the same as those described under quantitation of urinary metabolites.
All quantitative measurements were made relative to a constant amount of the internal standard, o-toluidine, and peak area ratios were used for the calculations. Standard curves, containing compounds of interest extracted from blank urine, were run on each day urine samples were analyzed (Tables 3 and 4). Quantitation was achieved graphically rather than by linear regression owing to the nonlinear response of some of the compounds. An authentic standard of 4-hydroxy-2,6-DMA was unavailable at the time of sample analysis; so 6-hydroxy-2,4-DMA was included in the appropriate standard curves as a substitute. Peak area ratios of the 6-hydroxy- to the 4-hydroxy-DMA standard were later used as a measure of the response factor to correct the calculated amounts of 4-hydroxy-2,6-DMA in each sample.

The amounts of parent compounds and metabolites conjugated with sulfate or glucuronic acid were calculated by methods of difference. The amount (mg) of compound present in the non-hydrolysed sample was subtracted from that measured in the sulfatase or glucuronidase treated sample. Occasionally, amounts present after enzymatic hydrolysis were less than that determined in the non-hydrolysed sample. This has been observed in other studies utilizing enzymatic hydrolysis. 105

Nuclear magnetic resonance analysis of the 6-hydroxy-2,4-DMA standard and 4-hydroxy-2,6-DMA isolated from rat urine was generously provided by Ethyl Corporation, Baton Rouge, LA.
Table 3. Metabolites Included in Standard Curves.

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Species</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-DMA</td>
<td>Rat</td>
<td>2,4-DMA, N,2,4-trimethylaniline, N-acetyl-4-amino-3-methyl-benzoic acid, methyl ester</td>
</tr>
<tr>
<td>2,4-DMA</td>
<td>Dog</td>
<td>2,4-DMA, N-2,4-trimethylaniline, 4-amino-3-methyl-benzoic acid, methyl ester, 6-hydroxy-2,4-dimethylaniline</td>
</tr>
<tr>
<td>2,6-DMA</td>
<td>Rat</td>
<td>2,6-DMA, N-2,6-trimethylaniline, 6-hydroxy-2,4-dimethylaniline</td>
</tr>
<tr>
<td>2,6-DMA</td>
<td>Dog</td>
<td>2,6-DMA, N,2,6-trimethylaniline, 2,6-dimethylnitrosobenzene, 2-amino-3-methyl-benzoic acid, methyl ester, 6-hydroxy-2,4-dimethylaniline</td>
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Table 4. Gas Chromatograph Retention Times of Standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Temp Program</th>
<th>Retention Time (min)</th>
<th>Temp Program</th>
<th>Retention Time (min)</th>
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</thead>
<tbody>
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<td>2,4-DMA</td>
<td>TP-1*</td>
<td>5.0</td>
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<tr>
<td>N,2,4-trimethylaniline</td>
<td>TP-1</td>
<td>5.6</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6-hydroxy-2,4-DMA</td>
<td>TP-1</td>
<td>6.5</td>
<td>TP-2**</td>
<td>11.3</td>
</tr>
<tr>
<td>4-amino-3-methyl-benzoic acid, methyl ester</td>
<td>TP-1</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-acetyl-4-amino-3-methyl-benzoic acid, methyl ester</td>
<td>TP-1</td>
<td>10.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,6-DMA</td>
<td>TP-1</td>
<td>5.0</td>
<td>TP-2</td>
<td>8.4</td>
</tr>
<tr>
<td>N,2,6-trimethylaniline</td>
<td>TP-1</td>
<td>6.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,6-dimethylnitrosobenzene</td>
<td>-</td>
<td>-</td>
<td>TP-2</td>
<td>7.5</td>
</tr>
<tr>
<td>4-hydroxy-2,6-DMA</td>
<td>TP-1</td>
<td>7.4</td>
<td>TP-2</td>
<td>10.3</td>
</tr>
<tr>
<td>2-amino-3-methyl-benzoic acid, methyl ester</td>
<td>-</td>
<td>-</td>
<td>TP-2</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Refers to Temperature Program 1 as described in the section intitled "Metabolite Quantification".

** Refers to Temperature Program 2 as described in the section intitled "Metabolite Quantification".
Covalent Binding of $^{14}$C-2,4-DMA and $^{14}$C-2,6-DMA to Hepatic DNA, RNA and Protein in the Rat

Isolation of Hepatic DNA

Hepatic DNA, RNA and protein were isolated by a modification of the methods of Markov and Ivanov,\textsuperscript{106} Casanova-Schmidt and D'A Heck,\textsuperscript{107} and Jollow, \textit{et al.}\textsuperscript{108} The pooled hepatic tissues of 4 rats were minced, suspended in 10 volumes of a lysing medium consisting of 9 M urea - 0.24 M NaPO$_4$, pH 6.8 - 0.01 M EDTA-1% sodium dodecyl sulfate (MUP-SDS) and homogenized with a Potter-Elvehjen tissue grinder. The homogenate was then subjected to further homogenization by explosive decompression using a cell bomb, in which the homogenate was held for 5 min at 700 PSI in an atmosphere of N$_2$ before release of pressure.\textsuperscript{109} To extract hepatic protein, an equal volume of chloroform - isoamyl alcohol-phenol (CIP), 24:1:25, was added to the lysate, agitated for 15 minutes on a wrist action shaker, and centrifuged on a clinical centrifuge (Sorvall GLC-2B General Laboratory Centrifuge, Du Pont Instruments, Newtown, CT) for 10 minutes at 2000 rpm at room temperature. The aqueous phase was removed and extracted again with CIP. In the event that the first CIP extraction resulted in a very small aqueous layer, the organic and interfacial phases were extracted with MUP-SDS, vortexed, and centrifuged on a clinical centrifuge. The aqueous phase was then treated as previously described. The organic phase was retained for analysis of precipitated protein.
The combined aqueous phases were extracted 3 times with an equal volume of ether to remove phenol. The organic phase was discarded and the aqueous layer applied to 2 hydroxylapatite columns (Bio Gel® HPT, Bio-Rad Laboratories, Richmond, CA) in sequence. The columns were prepared as follows: 4 g of hydroxylapatite (sufficient for 3 g of liver) were suspended in 40-50 ml of 0.01 M NaPO4, pH 6.8, boiled for 3 minutes, washed 3 times with the above buffer, and applied to a glass column (Econo-Column, 20 cm X 3 cm O.D., Bio-Rad Laboratories Richmond, CA). The column was washed with 0.02 M NaPO4, pH 6.8, and equilibrated with 2 bed volumes of 2 M urea - 0.24 M NaPO4, pH 6.8 (MUP). The first 6-7 ml eluting from the column, representing MUP from the equilibration process, were discarded. RNA and any contaminating protein were then removed from the column by eluting with MUP until UV absorbance at 260 nm equalled zero. The elutant was retained for RNA analysis and for analysis of contaminating protein.

Urea was eluted with 0.014 M NaPO4, pH 6.8; washing continued until the refractive index of the eluate equalled that of 0.014 M NaPO4. DNA was recovered from the column in 5 ml fractions by washing with 0.45 M NaPO4, pH 6.8, until the UV absorbance at 260 nm equalled zero. Fractions absorbing light at 260 nm were combined, the volume measured, and the total concentrated to 3.5 ml by ultrafiltration (Stirred Cell, 50 ml, YM 10 filter, Amicon, Danvers, MA). The concentrate was washed with 10 volumes of distilled water and reconcentrated. The DNA content was determined using an extinction coefficient of 20 ml/mg.cm at 260 nm for a solution of 1 mg/ml. An aliquot of 0.5 ml was added to 10 ml of scintillation fluor (Hydrofluor®, National Diagnostics, Somerville, NJ)
containing 0.5 ml tissue solubilizer (ScinitGest®, Fisher Scientific Co., Fair Lawn, NJ), vortexed, stored in the dark overnight, and counted for 50 minutes at a window setting of 90-620. (Model 6892 Beta Counter, Searle, Des Raines, IL). Results were expressed as the DNA covalent binding index and picomoles bound/mg DNA.

Isolation of Hepatic Protein

The organic phase of the CIP extraction was treated with 3 volumes of ethanol, vortexed, and centrifuged on a clinical centrifuge at 2000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet was suspended in 5 volumes of ethanol and centrifuged. Ethanol washes of the pellet were repeated until no radioactivity could be detected in the supernatant. All radioactivity remaining in the pellet was assumed to be covalently bound to protein. The pellet was dissolved in 1 N NaOH and an aliquot (50 μl) was counted by liquid scintillation as described for DNA. A second aliquot was subjected to protein determination, as previously described. Results were expressed as picomoles bound/mg protein.

Isolation of Hepatic RNA

The hydroxylapatite column eluates containing UV absorbing (260 nm) material were combined, concentrated to 3-5 ml by ultrafiltration, washed with 10 volumes of distilled water and reconcentrated. The absorbance was measured at 260 nm and the yield of RNA quantified as for DNA. Aliquots were counted by liquid scintillation as described for DNA.
and analyzed for protein content. Results were expressed as picomoles bound/mg RNA.

Calculation of Covalent Binding Index and Picomoles Bound/mg DNA, RNA or Protein

Samples of DNA, RNA or protein isolated from treated or non-treated (control) animals were counted via liquid scintillation for 50 minutes. Background counts were determined utilizing vials containing the scintillation cocktail only. Background radioactivity was subtracted from the sample counts, the counts converted to DPM by dividing by the counting efficiency and the counts converted to DPM/mg DNA, RNA or protein. The average DPM/mg for control samples was subtracted from the sample values. The DNA Covalent Binding Index (CBI)\textsuperscript{23} was determined using the following formulas:\textsuperscript{23}

\[
\text{CBI}^{-} = \frac{\text{DPM Chemical Bound/mg DNA}}{\text{DPM Chemical Administered/kg Body Weight}}
\]

\[
\text{CBI} = \frac{\text{CBI}^{-}}{3.24 \times 10^{-9}}
\]

Picomoles of chemical bound per mg DNA, RNA or protein were calculated using the following formulas:

\[
\mu\text{M Chemical Bound/} \frac{\text{mg DNA, RNA or protein}}{\text{mg DNA, RNA or protein}} = \frac{\text{DPM/mg}}{(2.2 \times 10^{9} \text{ DPM/Ci}) \times (1 \times 10^{3} \mu\text{Ci/mCi})}
\]

\[
= \frac{10.9 \mu\text{Ci} [^{14}\text{C}] - 2,6\text{-DMA/} \mu\text{Mole 2,6-DMA}}{10.9 \mu\text{Ci} [^{14}\text{C}] - 2,6\text{-DMA/} \mu\text{Mole 2,6-DMA}}
\]
pM Chemical Bound/ 
mg DNA, RNA or $= (\mu \text{Mole/mg})(1 \times 10^3 \text{nMole/mg})(1 \times 10^3 \text{pMole/mg})$

Statistical Analysis

Analysis of variance (General Linear Model) was used to determine differences due to treatment in rat body weights, liver weights and liver to body weight ratios. Scheffe's test was then used for multiple comparisons between the means of the different treatment groups for each of the above parameters. Differences were accepted at the ($p \leq 0.05$) level.

Analysis of variance and Dunnett's post test were used to compare the effects of PB, 3MC and SKF-525A on urinary excretory products of 2,4- and 2,6-DMA on Days 1 and 10 of treatment. A paired T test was used to compare the effect of length of treatment (Day 1 vs Day 10) on urinary excretory products within each rat and dog treatment group. Differences were accepted at the ($p \leq 0.05$) level.

An unpaired T test was used to compare the CBI and picomoles bound/mg DNA, RNA or protein between $^{14}\text{C}-2,4\text{-DMA}$ and $^{14}\text{C}-2,6\text{-DMA}$ treatment groups. Analysis of variance and Scheffe's post test were used to compare hepatic DNA, RNA or protein concentrations between controls, the $^{14}\text{C}-2,4\text{-DMA}$ treatment group and the $^{14}\text{C}-2,6\text{-DMA}$ treatment group. Differences were accepted at the ($p \leq 0.05$) level.
RESULTS

Hepatotoxicity

Clinical Observations in the Rat

Rats gavaged with either corn oil, 2,4-DMA or 2,6-DMA (Groups 9, 1, and 5, respectively) appeared clinically normal. Rats dosed with 3MC in addition to the above compounds (Groups 11, 3, and 7) also appeared healthy, except for an obvious weight loss. The combination of PB and 2,4-DMA (Group 2) proved lethal to 8 out of 16 animals by day 5. Clinical signs prior to death included weight loss, roughened hair coat, depression and chromodacryorrhea. PB:C (Group 10) and PB:2,6-DMA (Group 6a) rats; however, remained essentially normal up to Day 5. From Day 5 to 10, the PB:2,6-DMA (Group 6b) treatment group began showing clinical signs similar to those in the PB:2,4-DMA group, although less severe in degree. Rats in the SKF:Control, :2,4-DMA and :2,6-DMA (Groups 12, 4, and 8 respectively) groups exhibited weight loss, diarrhea, distended abdomens and rough hair coats. Animals treated with 2,4- or 2,6-DMA plus SKF appeared more seriously effected than the controls.

Various lesions were observed on necropsy. Adhesions were noted for all groups receiving IP injections and were most numerous in animals treated with SKF-525A. A cobblestoned liver surface was seen in rats treated with 2,4-DMA and PB:2,4-DMA. 3MC-dosed rats (Control and treatment
groups) had yellowish-white plaques scattered throughout the peritoneal cavity, and on the surfaces of the liver, diaphragm, omentum, and intestines. SKF-525A rats (Control and treated animals) exhibited evidence of peritonitis consisting of reddened omentum and small abscesses. The large intestine and cecum of these animals were bloated and filled with gas and fluid. Also, the adrenal glands were greatly enlarged and the livers were yellow in color and friable.

Only the combination of PB and 2,4-DMA produced significant mortality. As stated previously, eight out of sixteen animals in this group died between Days 1 and 5. One rat per group also died in the PB:C, SKF:C and SKF:2,4-DMA groups.

**Rat Body and Organ Weights**

Significant differences (p ≤ 0.05) due to treatment were detected in body weight, liver weight and the liver to body weight ratio on Days 5 and 10 (Tables 5 and 6). On Day 5 the combination of PB and 2,6-DMA decreased body weight when compared to PB control values, but induced no change in liver weight or liver weight ratio. Liver weight and the liver:body weight ratio were increased in the PB:2,4-DMA group as compared to PB:C and PB:2,6-DMA groups. On Day 10, there was no significant difference in body weight between corn oil gavaged controls, 2,4-DMA or 2,6-DMA groups. However, 2,4-DMA treatment increased liver weight and the liver to body weight ratio over the control and 2,6-DMA groups. Resembling its effect at 5 days, the combination of PB and 2,6-DMA at Day 10 depressed body weight, but did not alter liver weight.
Table 5. Body Weights, Liver Weights and Liver to Body Weight Ratios for Rats Sacrificed 24 Hours after 5 Consecutive Days of Dosing.

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Treatment</th>
<th>n</th>
<th>Body Wt.(g)</th>
<th>Liver Wt.(g)</th>
<th>Liver to body Wt. Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td>PB:C</td>
<td>8</td>
<td>260.63±5.29+</td>
<td>12.92±0.38</td>
<td>4.97±0.11</td>
</tr>
<tr>
<td>2</td>
<td>PB:2,4-DMA</td>
<td>8</td>
<td>256.63±9.21</td>
<td>15.31±1.00*</td>
<td>5.97±0.36*</td>
</tr>
<tr>
<td>6a</td>
<td>PB:2,6-DMA</td>
<td>8</td>
<td>249.75±6.51*</td>
<td>12.84±0.46</td>
<td>5.14±0.16</td>
</tr>
</tbody>
</table>

+ Mean ± standard deviation.
* Indicates values significantly different (P≤0.05) from PB:control.
Table 6. Body Weights, Liver Weights and Liver to Body Weight Ratios for Rats Sacrificed 24 Hours After 10 Consecutive Days of Dosing.

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Treatment</th>
<th>n</th>
<th>Body Wt.(g)</th>
<th>Liver Wt.(g)</th>
<th>Liver to Body Wt. Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Control</td>
<td>16</td>
<td>265.26±15.10+</td>
<td>10.58±1.20</td>
<td>3.98±0.33</td>
</tr>
<tr>
<td>1</td>
<td>2,4-DMA</td>
<td>16</td>
<td>267.10±11.66</td>
<td>14.53±1.06*</td>
<td>5.45±0.39*</td>
</tr>
<tr>
<td>5</td>
<td>2,6-DMA</td>
<td>16</td>
<td>266.43±9.15</td>
<td>10.77±1.28</td>
<td>4.04±0.45</td>
</tr>
<tr>
<td>10b</td>
<td>PB:C</td>
<td>7</td>
<td>257.74±9.29</td>
<td>12.51±0.49</td>
<td>4.86±0.23</td>
</tr>
<tr>
<td>6b</td>
<td>PB:2,6-DMA</td>
<td>8</td>
<td>231.76±8.24*</td>
<td>11.59±0.75</td>
<td>4.99±0.21</td>
</tr>
<tr>
<td>11</td>
<td>3MC:C</td>
<td>16</td>
<td>241.83±6.28</td>
<td>13.29±0.80</td>
<td>5.49±0.27</td>
</tr>
<tr>
<td>3</td>
<td>3MC:2,4-DMA</td>
<td>16</td>
<td>250.29±15.25</td>
<td>15.17±1.48*</td>
<td>6.05±0.35*</td>
</tr>
<tr>
<td>7</td>
<td>3MC:2,6-DMA</td>
<td>16</td>
<td>248.85±10.88</td>
<td>12.83±0.84</td>
<td>5.16±0.28</td>
</tr>
<tr>
<td>12</td>
<td>SKF:C</td>
<td>15</td>
<td>238.37±10.36</td>
<td>11.39±0.95</td>
<td>4.78±0.29</td>
</tr>
<tr>
<td>4</td>
<td>SKF:2,4-DMA</td>
<td>15</td>
<td>243.85±15.58</td>
<td>13.58±1.91*</td>
<td>5.57±0.31*</td>
</tr>
<tr>
<td>8</td>
<td>SKF:2,6-DMA</td>
<td>16</td>
<td>246.69±10.63</td>
<td>11.83±0.72</td>
<td>4.80±0.27</td>
</tr>
</tbody>
</table>

+ Mean ± standard deviation.
* Indicates values significantly different (P≤0.05) from the corresponding control group.
or the liver to body weight ratio when compared to the PB:C group. Treatment with 3MC or SKF-525A in conjunction with either of the dialkylanilines produced the same response as treatment with 2,4 or 2,6-DMA alone; that is, an increase in liver weight and liver to body weight ratios was induced by 2,4-DMA with no difference detected for 2,6-DMA (as compared to the corresponding control groups).

Treatment for 10 days with either 3MC or SKF-525A significantly decreased body weight when compared to corn oil gavaged controls. The PB: Control group was not significantly different from corn oil gavaged control animals. In addition, the body weights of the PB:2,6-DMA and SKF:2,4-DMA groups were significantly lower than the 2,6-DMA and 2,4-DMA treatment groups, respectively. No difference was noted between 3MC:2,6-DMA and 2,6-DMA, SKF:2,6-DMA and 2,6-DMA, or 3MC:2,4-DMA and 2,4-DMA at 10 days.

With respect to liver weight, treatment with PB or 3MC for 10 days produced a significant increase when compared to corn oil gavaged controls. SKF-525A did not produce a statistically significant change. There was no significant difference between treatment with 2,4-DMA, 3MC:2,4-DMA or SKF:2,4-DMA. Also, no differences between 2,6-DMA and PB:2,6-DMA or 2,6-DMA and SKF:2,6-DMA were found.

Liver to body weight ratios were significantly increased over corn oil gavaged controls on Day 10 by PB, 3MC and SKF-525A. The combination of 3MC and 2,4-DMA increased the ratio over 2,4-DMA animals, whereas SKF-525A given in conjunction with 2,4-DMA did not. Treatment with
either PB, 3MC or SKF-525A concurrently with 2,6-DMA increased liver to body weight ratios over animals treated solely with 2,6-DMA.

In summary 2,4-DMA, whether administered alone or in conjunction with PB, 3MC or SKF-525A, consistently increased liver and liver to body weight ratios over corresponding control and 2,6-DMA animals. Likewise, 2,6-DMA did not alter mean liver weights or percent liver of body weight from control values. Body weight, however, was significantly decreased from PB:C by the combination of phenobarbital and 2,6-DMA on Days 5 and 10. 3MC and SKF-525A, at 15 mg/kg and 50 mg/kg, respectively, for 10 days induced toxicity as evidenced by the decrease in mean body weights from corn oil gavaged controls.

Histopathologic Evaluation in the Rat

Light microscopic examination of livers from corn oil gavaged controls (Group 9) and animals treated with 2,6-DMA (Group 5) were within the range of normal (Figure 15). Hepatocytes in rats treated with 2,4-DMA (Group 1) showed a distinct, but subtle, change. The cells were enlarged and the sinusoids less prominent. The voluminous cytoplasm was paler staining and more homogenous than that found in controls. Overlaying the pale cytoplasm were dark staining basophilic structures of irregular outline. This change was thought to be due to segregation or clumping of subcellular organelles (Figure 16). The cell outline was not distinct and nuclei appeared larger than normal. One section contained an average of 2 mitotic figures per high power field.
The appearance of livers from rats treated for 5 or 10 days with phenobarbital or phenobarbital and 2,6-DMA (Groups 10a, 10b, 6a, and 6b) on light microscopy was similar. Cells were enlarged and a sinusoidal collapse was seen. Very fine, scattered vacuoles of lipid were noted (Figure 17). Confirmation of lipid content was obtained on frozen sections stained with Oil Red O. Changes observed in rats treated with phenobarbital and 2,4-DMA (Group 2) for 5 days were similar to those in rats treated with 2,4-DMA for 10 days (Group 1). Enlarged cells with a homogeneous cytoplasm containing segregated organelles were found (Figure 18). A large area of coagulative necrosis walled off by inflammatory response was found at the tip of the lobe in 4 out of 8 animals. This change was also found in 2 of 6 animals in group 6a and 1 of 7 animals in Group 6b. It is suggested that the necrosis was related to phenobarbital injection.

Changes observed on light microscopy of livers from rats treated with 3MC and gavaged with corn oil (Group 11) consisted of a collapse of the sinusoids, cell enlargement and a mild accumulation of fine scattered vacuoles of lipid (Figure 19). Lesions were similar in rats treated with 3MC and 2,6-DMA (Group 7), although lipid accumulation was slightly greater (Figure 19). The cytoplasmic change observed in animals treated with 2,4-DMA (Group 1) was more pronounced in rats receiving 2,4-DMA and 3MC (Group 3) (Figure 20).

Rats treated with SKF (Group 12) for 10 days developed a mild to severe midzonal fatty degeneration. Multiple vacuoles of varying sizes filled the cytoplasm of affected cells. Lipid vacuoles in SKF treated animals
Figure 15. Histologic appearance of rat liver following treatment with corn oil (A) or 2,6-DMA (B). Both sections were considered to be within the range of normal. 290 x.
Figure 16. Hepatic effect of 2,4-DMA in the rat. Note the enlarged cells and the dark staining irregular structures scattered throughout the cytoplasm. 290 x.
Figure 17. Hepatic effect of PB (a) and PB in conjunction with 2,6-DMA (b). 450 x.
Figure 18. Hepatic effect of treatment with 2,4-DMA in conjunction with PB. Note the similarity to Figure 16. 450 x.
Figure 19. Hepatic effect of 3MC (a) and 3MC in conjunction with 2,6-DMA (b). A mild accumulation of lipid is visible. 450 x.
Figure 20. Hepatic effect of treatment with 2,4-DMA in conjunction with 3MC. The cytoplasmic change observed in animals treated with 2,4-DMA alone (Figure 16) is more pronounced. 450 x.
Figure 21. Hepatic effects of SKF-525A.  

A. Midzonal fatty degeneration, 115 x.  

B. Higher magnification of A showing lipid accumulation in circular vacuoles, 450 x.
Figure 22. Hepatic effect of concurrent treatment with SKF-525A and 2,6-DMA. The midzonal fatty degeneration is less prominent than in Figure 21 A. 115 x.
Figure 23. Hepatic effect of concurrent treatment with SKF-525A and 2,4-DMA. A. Absence of midzonal fatty degeneration, 115 x. B. Higher magnification of A showing two cells in which lipid accumulation is evident; for comparison to Figure 21 B. 450 x.
were circular in nature as opposed to the irregular form found in other rat treatment groups and dogs treated with 2,6-DMA (Figure 21). A general decrease in the size of the sinusoids was observed, except for the area immediately surrounding central veins. Mitotic figures were found on several sections.

Concurrent treatment with SKF and 2,6-DMA (Group 8) lessened the severity of the midzonal fatty degeneration (Figure 22). Animals in the SKF:2,4-DMA group (Group 4) exhibited no (or minimum) lipid accumulation (Figure 23). The segregation of cellular organelles which was characteristic of 2,4-DMA cytoplasm was not noticeable in this group.

**Electron Microscopic Evaluation in the Rat**

Electron microscopic examination of livers from 2,4-DMA treated rats revealed proliferation of smooth endoplasmic reticulum (SER), occasional small fat vacuoles, and segregated islands of rough endoplasmic reticulum (RER) (Figure 24). The islands of RER contained the majority of RER within the cell and were composed of straight segments of lamellae. Generally, the islands were located near a group of mitochondria and bordered on at least one side by these organelles. In contrast, control animals displayed a more curvilinear form of RER which was scattered randomly throughout the cytoplasm (Figure 25). Livers from rats treated with 2,6-DMA were within the range of normal.

Phenobarbital treatment induced a proliferation of SER as expected. Membrane-bound lipid vacuoles of varying sizes were also noted in some
cells, as were aggregates of polyribosomes. Islands of RER were seen, but this change was not as prominent as in the 2,4-DMA treatment group (Figure 26). The combination of phenobarbital and 2,4-DMA produced proliferation and dilatation of SER, small fat vacuoles of varying size and linear arrangement of RER. In general, the amount of RER and numbers of mitochondria appeared to have decreased. The mitochondria appeared normal in configuration, however. Numerous lysosomes and several dilated Golgi organs were observed. No unusual changes were observed in bile canaliculi (Figure 27).

Changes observed in the livers of rats treated with phenobarbital and 2,6-DMA included hyperplasia of the SER, proliferation of lysosomes and variation in mitochondrial size. Lipid inclusions ranging in size from very fine membrane-bound vesicles to large non-bound vacuoles which displaced the nucleus were also frequently observed. Several dilated Golgi organs were seen (Figure 28).

The segregation or packing of RER into islands was very pronounced in rats treated with 3MC and 2,4-DMA. This change was enhanced over that seen in the 2,4-DMA treatment group. The lamellae of the RER were widely spaced with considerable dark-staining granular material within. This dark-staining material may represent proteinaceous products elaborated by the ribosomes of the RER. Aggregates of polyribosomes were frequently observed. Hyperplasia of SER accompanied this change in RER structure. Normal numbers of mitochondria and an occasional fat globule were seen (Figure 29). The combined effects of SER hyperplasia, polyribosome aggregation, and RER islands were felt to be responsible
Figure 24. Hepatic ultrastructural changes in an animal treated with 2,4-DMA. A. Segregated islands of RER and lipid vacuoles. B. Proliferation of SER. 12500 x.
Figure 25. Hepatic ultrastructure of a control animal. A. Random arrangement of RER, 4900 x. B. Curvilinear RER, 10000 x.
Figure 26. Hepatic ultrastructure following treatment with PB.

A. Proliferation of SER, islands of RER and small lipid vacuoles, 10000 x.  
B. Aggregate of polyribosomes, 12500 x.
Figure 27. Hepatic ultrastructure following treatment with 2,4-DMA in conjunction with PB. A. Note lysosomes and proliferation of SER, 12500 x. B. Higher magnification of A showing dilation of SER, 16500 x.
Figure 28. Hepatic ultrastructure following treatment with 2,6-DMA in conjunction with PB. A. Proliferation of SER and variation in mitochondrial size, 10000 x. B. Higher magnification of A, 12500 x.
Figure 29. Hepatic ultrastructure following treatment with 2,4-DMA in conjunction with 3MC. A. Segregation of RER and widely spaced lamellae, 10000 x. B. Higher magnification of A, 12500 x.
Figure 30. Nonspecific degenerative changes following treatment with SKF-525A.  
A. Lysosomes and early calcification of mitochondria, 10000 x.  
B. Lipid accumulation, 6200 x.
for the subtle 2,4-DMA cytoplasmic lesion seen on light microscopy. Electron microscopic hepatic changes in rats treated with 3MC and 2,6-DMA were similar to those described above, although fat vacuoles were more numerous and the RER segregation less pronounced.

Electron microscopy of the SKF groups (Control, 2,4-DMA, and 2,6-DMA) showed nonspecific degenerative changes (Figure 30). Numerous lysosomes, membrane-bound fat globules and early calcification of mitochondria were seen. Lipid accumulation, at times displacing the nucleus and most of the cytoplasm, was most severe in livers from the SKF:Control group. Aggregation of RER was not seen in any SKF treatment group.

Clinical Observations in the Dog

Oral administration of 2,4- or 2,6-DMA induced vomiting in one dog in each of the treatment groups (dog numbers 2,4-1 and 2,6-1) on Day 1. Productive vomiting was not observed in the remaining dogs, although retching and excessive salivation was seen immediately post-dosing.

After 10 days of continuous dosing, all dogs in both groups appeared unthrifty with a loss of gloss to the haircoat. Dogs treated with 2,6-DMA were most severely affected in this respect. All dogs were active, bright and alert, however. Animals in the 2,6-DMA and 2,4-DMA treatment groups sustained a mean weight loss of 2.38 ± 0.48 kg and 0.68 ± 0.40 kg, respectively.
Visual observation of the liver during biopsy on Day 11 revealed an enlarged, pale, blunt-edged organ in all dogs treated with 2,6-DMA. Only one dog showed clinical evidence of icterus (yellow-tinged sclera), however. Livers of dogs receiving 2,4-DMA appeared normal on visual inspection.

Histopathologic and Electron Microscopic Evaluation in the Dog

Light microscopic evaluation of liver biopsies from dogs treated with 2,6-DMA revealed moderate to severe centrilobular fatty degeneration. The fatty degeneration was panlobular in distribution in one dog. Effected hepatocytes were enlarged and the cytoplasm was filled with large, clear vacuoles (Figure 31). Confirmation of the lipid content within the vacuoles was obtained with frozen sections stained with Oil Red O. Control biopsies taken from the dogs prior to treatment with 2,6-DMA showed no evidence of fatty degeneration and were within the range of normal (Figure 32).

The most prominent electron microscopic change in the livers of dogs treated with 2,6-DMA was the accumulation of lipid. This accumulation was much more severe than that seen in any of the rat treatment groups and consisted of multiple membrane-bound vesicles of varying sizes. These vesicles often displaced the cytoplasm and/or nucleus. Dilated SER was also observed (Figures 33 and 34).

In contrast to the effect of 2,6-DMA, liver biopsies from dogs treated with 2,4-DMA showed no evidence of fatty degeneration on light
Figure 31. Hepatic effect of 2,6-DMA in the dog. A. Centrilobular fatty degeneration, 290 x. B. Higher magnification of A, showing irregularly shaped lipid vacuoles, 450 x.
Figure 32. Control liver biopsy prior to treatment with 2,6-DMA.  450 x.
Figure 33. Hepatic ultrastructure of control animal. A. 1950 x.
B. 4900 x.
Figure 34. Hepatic effect of 2,6-DMA in the dog at the ultrastructural level.  

A. Membrane-bound lipid vacuoles, 4900 x.  

B. Dilation of SER, 16500 x.
Figure 35. Photomicrograph of liver biopsy following treatment with 2,4-DMA. 450 x.
microscopic evaluation. Stained sections of liver biopsies taken before and after dosing with 2,4-DMA were within the range of normal (Figure 35). No ultrastructural lesions were detected on electron microscopic evaluation.

**Urinary Metabolites**

**Metabolite Identification**

The extraction and chromatographic conditions described under "Metabolite Isolation and Identification - Preliminary Study" adequately separated normal urine constituents, the enzyme modifiers PB and SKF-525A and a majority of standards. The extraction process and temperature program used for 2,4-DMA samples in the preliminary study achieved extraction and chromatographic separation of the following standards: o-toluidine, 2,4-DMA, N-acetyl-2,4-DMA, 2,4-dimethyl-nitrosobenzene, 2-amino-5-methyl-benzoic acid (methyl ester), and 4-amino-3-methyl-benzoic acid (methyl ester). The extraction process and temperature program used for 2,6-DMA samples in the preliminary study extracted and separated o-toluidine, 2,6-DMA, N-acetyl-2,6-DMA, 6-hydroxy-2,4-DMA, 4-hydroxy-2,6-dimethylnitrobenzene and 2-amino-3-methyl-benzoic acid, methyl ester. N-hydroxy-2,6-DMA, 4-N-hydroxy-imino-3,5-dimethyl-quinone and 3MC could not be detected with the chromatographic system in use. Standards N,2,4-trimethylaniline, 4-hydroxy-2,6-DMA, N,2,6-trimethylaniline, N-acetyl-4-amino-3-methyl-benzoic acid, 2-amino-3-methyl-benzyl alcohol
and 2-amino-3-methyl-benzaldehyde were not available at the time of the preliminary study.

Metabolites present in the urine of treated dogs and rats were identified on the basis of the similarity of their GC retention times and mass spectra to authentic standards. When an authentic standard was unavailable, a chemical structure was proposed based on the interpretation of the analyte's mass spectra. Metabolites for which standards were prepared but which were not detected in urine included N-acetyl-2,6-DMA, N-acetyl-2,4-DMA, 2,6-dimethyl-nitrobenzene, 6-hydroxy-2,6-dimethyl-nitrobenzene, N-hydroxy-2,6-DMA, 2-amino-5-methyl-benzoic acid, 4-N-hydroxy-imino-3,5-dimethyl-quinone, 2,4-dimethyl-nitrosobenzene, 2-amino-3-methyl-benzyl alcohol, and 2-amino-3-methyl-benzaldehyde. A comparison of the mass spectra of metabolites and standards is presented in Table 7. Interpretations of mass spectra are presented in the following paragraphs.

2,4-DMA. An intense molecular ion (m/z 121) was produced by 2,4-DMA as would be expected for an aromatic amine (Table 7).\textsuperscript{111} A loss of one proton would produce the fragment at m/z 120 (M-1\textsuperscript{+}). The fragment at m/z (M-15\textsuperscript{+}) was interpreted as the loss of a ring methyl group, common in xylenes. The fragment at m/z 91 may represent a tropilium ion, C\textsubscript{7}H\textsubscript{7}\textsuperscript{+}, which is indicative of an alkyl substituted benzene ring, or may be the result of the loss of both ring methyl groups (C\textsubscript{6}H\textsubscript{3}NH\textsubscript{2}\textsuperscript{+}). A cluster of peaks at m/z 77, observed in the mass spectrum, is common in alkylbenzene compounds.\textsuperscript{111}
Table 7. Comparison of the Mass Spectra of Chemical Standards and Compounds Isolated from the Urine of Dogs and Rats Treated with 2,4- or 2,6-DMA.

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2,6-DMA. This compound gave a mass spectrum that was identical to its isomer, 2,4-DMA (Table 7).

6-Hydroxy-2,4-DMA. A compound matching the retention time and mass spectrum of the authentic standard of 6-hydroxy-2,4-DMA was observed in the urine of dogs treated with 2,4-DMA (Table 7). A conspicuous parent peak, noted here at m/z 137, is a common characteristic of both aromatic amines and phenols. Similar to the mass spectrum of 2,6-DMA, prominent M-1\(^+\) (m/z 136) and M-15\(^+\) (m/z 122) peaks were observed. Loss of the ring hydroxyl from the parent molecule would produce the ion at m/z 120. Peaks resulting from the loss of \(\cdot\text{CHO}\) (M-29\(^+\)) are common in phenols; it is probable that the fragment at m/z 108 represents this loss.

4-Hydroxy-2,6-DMA. A compound, which produced a mass spectrum virtually identical to that of 6-hydroxy-2,4-DMA, but exhibited a different gas chromatographic retention time, was detected in the urine of rats and dogs treated with 2,6-DMA (Table 7). An amount sufficient for proton nuclear magnetic resonance analysis was isolated and purified from the urine of treated rats. Results were consistent with an aromatic ring substituted by equivalent methyl groups (Figure 36). Nuclear magnetic resonance analysis of the 6-hydroxy-2,4-DMA standard indicated two unequivalent methyl groups (Figures 37 and 38). 2-Amino-3-methyl-benzyl alcohol, which could possibly arise from the metabolism of 2,6-DMA and which has a molecular weight equal to 4-hydroxy-2,6-DMA, was excluded on the basis of mass spectrum and retention time (Table 7). Therefore, it was concluded that the structure of the compound isolated from the
Figure 36. Proton nuclear magnetic resonance analysis of the 4-hydroxy-2,6-DMA chemical standard isolated from rat urine.
Figure 37. Proton nuclear magnetic resonance analysis of the 6-hydroxy-2,4-DMA chemical standard.
Figure 38. $^{13}$C-Nuclear magnetic resonance analysis of the 6-hydroxy-2,4-DMA chemical standard.
urine of treated dogs and rats was consistent with that of 4-hydroxy-2,6-DMA.

**N-acetyl-4-amino-3-methyl-benzoic acid, methyl ester.** A metabolite with a mass spectrum corresponding to the mass spectrum of a methyl ester of N-acetyl-4-amino-3-methyl-benzoic acid standard was observed in the urine of rats treated with 2,4-DMA (Table 7). The parent peak (m/z 207) was prominent as would be expected for a methyl ester of an aromatic carboxylic acid. Elimination of $\cdot\text{CH}_2\text{CO}$ from the parent molecule, a frequent loss for acetylated compounds, would produce the peak at m/z 165 (M-42$^+\)). A strong peak at m/z 43 substantiates the presence of an acetyl group. Further losses of $\cdot\text{OCH}_3$ and $\cdot\text{COOCH}_3$ could result in fragments m/z 134 and 106. Losses of this type are indicative of aromatic carboxylic acid esters.

The above compound could be differentiated from its isomer, the methyl ester of N-acetyl-2-amino-5-methyl-benzoic acid, by differences in retention time and mass spectra (Table 7).

**2-Amino-3-methyl-benzoic acid, methyl ester.** A metabolite corresponding to the mass spectrum of the above compound was found in the urine of dogs treated with 2,6-DMA (Table 7). A strong parent peak was observed, followed by a large peak at M-32$^+$. Elimination of $\cdot\text{CH}_3\text{OH}$ from the parent molecule would explain the prominent ion seen at m/z 133. Esters of aromatic acids may sustain such a loss if a hydrogen-bearing ortho group is available. Further elimination of $\cdot\text{CO}$ would then produce the fragment at m/z 105.
4-Amino-3-methyl-benzoic acid, methyl ester. A metabolite corresponding to the mass spectrum of the above compound was observed in the urine of dogs treated with 2,4-DMA (Table 7). The elimination pattern closely followed that of the methyl ester of N-acetyl-4-amino-3-methyl-benzoic acid. Losses of $\cdot$OCH$_3$ and $\cdot$COOCH$_3$ from the parent molecule were significant.

N,2,4-trimethylaniline. Urine of dogs and rats treated with 2,4-DMA contained a metabolite which corresponded to that of N,2,4-trimethylaniline (Table 7). As this compound may have resulted from the diazomethane derivatization and not via metabolic incorporation, a 2,4-DMA standard was reacted with diazomethane. No methylation of 2,4-DMA was observed, either by GC or GC/MS.

A strong parent peak (m/z 135) was observed followed by the loss of M-1$^+$, M-15$^+$, and M-30$^+$. The peaks at M-15$^+$ and M-30$^+$ were construed as consecutive losses of methyl groups.

N,2,6-trimethylaniline. Using selected ion monitoring of extracts of rat and dog urine, a compound with a molecular ion of 135 was found to coelute with 2,6-DMA. No additional fragments, other than those common to 2,6-DMA, were detected. It was concluded that methylation of 2,6-DMA, as with 2,4-DMA, had occurred in vivo to a minor extent.

2,6-Dimethylnitrosobenzene. A metabolite corresponding to the mass spectrum of 2,6-dimethylnitrosobenzene was observed in the urine of dogs treated with 2,6-DMA (Table 7). A strong parent peak (m/z 135) was seen
followed by the loss of 30 (\textsuperscript{14}CN\textsubscript{0}) to produce the fragment m/z 105. Clusters of peaks surrounding each of the major ions, common to aromatic nitrosoamines, were observed.

2-Amino-3-methyl-benzoic acid, methyl ester of the glycine conjugate. No authentic standard of the above compound was available. However, a compound with a mass spectrum which could be interpreted as the above compound was detected in the urine of dogs treated with 2,6-DMA (Table 7). The presence of 2-amino-3-methyl-benzoic acid in the urine of these dogs and the frequent conjugation of carboxylic acids with glycine lends support to this interpretation.

The ion at m/z 222 was considered to be the parent molecule and corresponds to the molecular weight of the methyl ester glycine conjugate of 2-amino-3-methyl-benzoic acid (Table 7). M-88\textsuperscript{+} (m/z 134) was interpreted as the loss of the fragment NHCH\textsubscript{2}COOCH\textsubscript{3}, consisting of the glycine methyl ester conjugate. A further loss of 29 (\textsuperscript{14}CO) would produce the fragment m/z 105.

4-Amino-3-methyl-benzoic acid, methyl ester of the glycine conjugate. An authentic standard of the above compound was not available, however a compound with a mass spectrum which could be interpreted as the above structure was isolated from the urine of dogs treated with 2,4-DMA (Table 7). Whether the original oxidation of a ring methyl to -COOH and subsequent conjugation with glycine occurred at the 2 or 4 position of 2,4-DMA is unclear. However, as 4-amino-3-methyl-benzoic acid was detected in the urine of these dogs it seems likely that the unknown is
the glycine conjugate of this compound and not its isomer, 2-amino-5-methyl-benzoic acid. A fragmentation pattern similar to that of the methyl ester glycine conjugate of 2-amino-3-methyl-benzoic acid is proposed.

Unknown metabolite, molecular weight of 135. A compound with an apparent molecular weight of 135 was observed in the urine of dogs treated with 2,6-DMA (Table 7). The mass spectrum of the unknown did not correlate with any standard on hand. Four possible structures of molecular weight 135 were proposed: 4-imino-3,5-dimethylquinone, its isomer 3-imino-2,4-dimethyl-quinone, 2,6-dimethyl-nitrosobenzene and 2-amino-3-methyl-benzaldehyde. 2,6-Dimethyl-nitrosobenzene and 2-amino-3-methyl-benzaldehyde were eliminated on the basis of retention times and mass spectra (Table 7). The formula 4-imino-2,6-dimethyl-quinone would appear to be the best choice for the unknown. 4-Hydroxy-2,6-DMA is a major metabolite of 2,6-DMA in the dog; hydroxylation occurring at the proposed location of the quinone's oxygen. Quinone formation could proceed in vivo or in vitro from the hydroxy compound. The possibility that the oxygen may be located meta to the nitrogen cannot be ruled out, but appears unlikely considering the metabolism of related compounds. 38-42

Urinary Metabolites of 2,4-DMA in the Rat

2,4-DMA was excreted in the urine of treated rats as the parent compound, N-acetyl-4-amino-3-methyl-benzoic acid and the sulfate or glucuronide conjugates of these compounds. N,2,4-trimethylaniline was
detected in the preliminary study, but at levels which were too low to allow quantitation (Figure 39 and Table 8).

In rats receiving 2,4-DMA only (Group 1) N-acetyl-4-amino-3-methyl-benzoic acid was the major excretory product on both Day 1 and Day 10 of treatment. A variable amount of glucuronidation was observed on Day 1, but none on Day 10. The sulfate conjugate was detected in one urine aliquot on Day 1 only. The parent compound was excreted free and as the glucuronide conjugate in roughly equal amounts on Day 1. At Day 10, glucuronidation had increased slightly or remained the same as on Day 1. Sulfation of the parent compound occurred to a minor extent on Day 1 with a slight increase on Day 10. There was no statistical difference on Day 10 as compared to Day 1 in the total excretion as parent compound, N-acetyl-4-amino-3-methyl-benzoic acid or the ratio of metabolite to parent compound (Table 9c).

Animals receiving PB and 2,4-DMA (Group 2) also excreted the benzoic acid derivative as the major urinary product of 2,6-DMA on Days 1 and 5 (Table 8). On Day 1 only one urine aliquot contained a sulfate or glucuronic acid conjugate of the metabolite. By day 5 the metabolite was excreted totally in the free form. The parent compound was excreted on Day 1 in the free form and as the sulfate and glucuronide conjugates. Excretion of the parent compound in the free form and as the glucuronide conjugate predominated. By Day 5 sulfate conjugation had increased over that observed for the glucuronic acid moiety. No statistical differences were noted in levels of parent, metabolite or the metabolite
Figure 39. Structures of the excretory products of 2,4-DMA detected in the urine of treated rats.
Table 8. Sum of all Forms (free, sulfate conjugate, glucuronide conjugate) of 2,4-DMA Excretory Products Isolated from the Urine of Rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (Total Mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
<th>2,4-DMA (Total Mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(2,4-DMA)</td>
<td>7.27±2.53**</td>
<td>19.07±5.29</td>
<td>3.21±2.45</td>
<td>0.06±0.02</td>
<td>0.09±0.03</td>
<td>2.01±1.53</td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>5.54±3.70</td>
<td>11.25±5.67</td>
<td>3.04±2.12</td>
<td>0.05±0.03</td>
<td>0.06±0.03</td>
<td>1.91±1.33</td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>3.79±1.25</td>
<td>31.71±14.81</td>
<td>8.06±2.13</td>
<td>0.03±0.01</td>
<td>0.15±0.07</td>
<td>5.06±1.34</td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>12.56±8.96</td>
<td>32.07±13.37</td>
<td>3.77±2.88</td>
<td>0.10±0.07</td>
<td>0.16±0.07</td>
<td>2.36±1.80</td>
</tr>
</tbody>
</table>

Day 5

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (Total Mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
<th>2,4-DMA (Total Mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(2,4-DMA)</td>
<td>-+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>5.18±5.80++</td>
<td>13.08±14.72</td>
<td>7.00±10.07</td>
<td>0.04±0.04</td>
<td>0.06±0.07</td>
<td>4.38±6.32</td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>-+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>11.63±6.91</td>
<td>81.42±23.49</td>
<td>8.25±4.01</td>
<td>0.09±0.05</td>
<td>0.42±0.12</td>
<td>5.1±2.5</td>
</tr>
</tbody>
</table>

Day 10

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (Total Mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
<th>2,4-DMA (Total Mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(2,4-DMA)</td>
<td>10.15±3.73</td>
<td>20.28±15.04</td>
<td>1.89±0.76</td>
<td>0.08±0.03</td>
<td>0.11±0.08</td>
<td>1.18±0.47</td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>-+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>9.32±4.26</td>
<td>29.65±14.68</td>
<td>3.11±0.66</td>
<td>0.08±0.04</td>
<td>0.15±0.07</td>
<td>1.94±0.41</td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>28.52±18.96</td>
<td>18.25±2.05</td>
<td>0.89±0.59</td>
<td>0.24±0.16</td>
<td>0.09±0.01</td>
<td>0.56±0.37</td>
</tr>
</tbody>
</table>

* Metabolite to parent Ratio
** Mean ± standard deviation
+ Urine was not collected from these treatment groups on day 5.
++ Represents quantity present in the urine of 8, rather than 16, rats.
+++ No animals in this group survived beyond Day 5.
### Table 9a. Statistical Comparisons of Groups 2, 3 and 4 Means to Group 1 Means on Day 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (Total mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total mg</td>
<td>Total mM</td>
<td></td>
</tr>
<tr>
<td>1(2,4-DMA)</td>
<td>7.27±2.53</td>
<td>0.06±0.02</td>
<td></td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>5.54±3.70</td>
<td>0.05±0.03</td>
<td></td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>3.79±1.25</td>
<td>0.03±0.01</td>
<td></td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>12.56±8.96</td>
<td>0.10±0.07</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates a significant difference (P<0.05)

### Table 9b. Statistical Comparisons of Groups 2, 3 and 4 Means to Group 1 Means on Day 10.

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (Total mg Excreted)</th>
<th>NAcCOOH (Total mM Excreted)</th>
<th>M/P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total mg</td>
<td>Total mM</td>
<td></td>
</tr>
<tr>
<td>1(2,4-DMA)</td>
<td>10.15±3.73</td>
<td>0.08±0.03</td>
<td></td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>9.32±4.26</td>
<td>0.08±0.04</td>
<td></td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>28.52±18.96</td>
<td>0.24±0.16</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates a significant difference (P<0.05)

+ Metabolite to parent ratio (mg or mM metabolite ÷ mg or mM parent).

++ No animals in this group survived beyond day 5.
Table 9c. Statistical Comparisons of Day 1 vs Day 10 Means for Groups 1, 2, 3 and 4.

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (mg) Day 1 vs. Day 10</th>
<th>NAcCOOH (mg) Day 1 vs. Day 10</th>
<th>M/P (mg) Day 1 vs. Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(2,4-DMA)</td>
<td>7.27±2.53</td>
<td>19.07±5.29</td>
<td>3.21±2.45</td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>5.54±3.70</td>
<td>11.25±5.67</td>
<td>3.04±2.12</td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>3.79±1.25</td>
<td>31.71±14.81</td>
<td>8.06±2.13</td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>12.56±8.96</td>
<td>32.07±13.37</td>
<td>3.77±2.88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>2,4-DMA (mM) Day 1 vs. Day 10</th>
<th>NAcCOOH (mM) Day 1 vs. Day 10</th>
<th>M/P (mg) Day 1 vs. Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(2,4-DMA)</td>
<td>0.06±0.02</td>
<td>0.09±0.03</td>
<td>2.01±1.53</td>
</tr>
<tr>
<td>2(PB:2,4-DMA)</td>
<td>0.05±0.03</td>
<td>0.06±0.03</td>
<td>1.91±1.33</td>
</tr>
<tr>
<td>3(3MC:2,4-DMA)</td>
<td>0.03±0.01</td>
<td>0.15±0.07</td>
<td>5.06±1.34</td>
</tr>
<tr>
<td>4(SKF:2,4-DMA)</td>
<td>0.10±0.07</td>
<td>0.16±0.07</td>
<td>2.36±1.80</td>
</tr>
</tbody>
</table>

* Indicates a significant difference (P<0.05)
+ Metabolite to parent ratio (mg or mM metabolite ÷ mg or mM parent).
++ No animals in this group survived beyond day 5.
to parent ratio for Group 2 when compared to Group 1 on Day 1 (Table 9a). It should be noted that results in the PB:2,4-DMA treatment group were highly variable (Table 8). The variability may have been related to the toxicity which produced death in 50% of the treatment group by Day 5.

Animals in Group 3 (3MC:2,4-DMA) also produced N-acetyl-4-amino-3-methyl-benzoic acid as the major excretory product of 2,4-DMA on Days 1 and 10. The metabolite was excreted primarily in the free form with no sulfate conjugation detected on either Day 1 or 10 of treatment and glucuronide conjugation was detected in only one aliquot on Day 1. Parent compound was excreted primarily in the free form with sulfation or glucuronidation occurring to a minor extent on Days 1 and 10.

Treatment with 3MC appeared to cause decrease and increase on Day 1 in the excretion as parent compound and the benzoic acid derivative, respectively, when compared to Group 1 (Table 9a). No statistical difference was detected, owing to the high variability. The metabolite:parent ratio was, however, significantly different from that of Group 1 on Day 1 (Table 9a). By Day 10 levels of the parent compound and the benzoic acid derivative in Group 3 were similar to those of Group 1 and no statistical differences were detected (Table 9b). The metabolite to parent ratio of Group 3 on Day 10 was significantly different from the value found on day 1 (Table 9c).
N-acetyl-4-amino-3-methyl-benzoic acid was again the major urinary excretory product isolated from animals receiving SKF-525A and 2,4-DMA (Group 4). The metabolite was primarily excreted in the free form. Evidence for glucuronidation was found in only one urine aliquot on Day 1 and in none of the samples on Days 5 and 10. Only a minor degree of sulfate conjugation was found on Day 10, and none was found on Days 1 and 5. Parent compound was excreted free and as the sulfate and glucuronide conjugates on Days 1, 5 and 10. The free form predominated on Days 1 and 5, whereas the sulfate and glucuronide conjugates predominated on Day 10.

No significant differences existed between Groups 1 and 4 on Days 1 and 10 of treatment (Tables 9a and 9b). Furthermore, there was no difference in the levels of parent, metabolite or the parent:metabolite ratio on Day 1 vs Day 10 for Group 4 (Table 9c). However, treatment with SKF-525A did appear to slightly increase the excretion as parent compound on both Day 1 and 10 (Tables 9a and 9b).

In summary, the metabolic pathway of 2,4-DMA was not altered by 1 or 10 days of treatment or by concurrent treatment with PB, 3MC or SKF-525A. N-acetyl-4-amino-3-methyl-benzoic acid was the major excretory product in all groups and on all days of treatment. The metabolite was excreted primarily in the free, rather than conjugated, form. Elimination of the parent compound as the free or conjugated form was variable and dependent on treatment group and duration of treatment.
Few statistical differences due to the length of treatment or concurrent administration of PB, 3MC or SKF-525A were observed. Calculated values for the amounts of parent compound and metabolite present in urine or for the metabolite to parent ratio were highly variable. As these values were much more consistent in animals treated with 2,6-DMA, the variability may be related to the toxicity of 2,4-DMA. A direct inspection of the data without regard to statistical differences reveals certain trends, however. PB appeared to decrease the urinary elimination of 2,4-DMA as either the parent compound or as the benzoic acid derivative on Day 1 when compared to animals receiving 2,4-DMA alone. This trend appeared to continue on Day 5. 3MC on the other hand, appeared to enhance the production of the benzoic acid derivative as compared to animals receiving 2,4-DMA alone and to therefore decrease the quantity of parent compound detected in the urine. SKF-525A tended to increase the quantity detected as parent compound on Days 1 and 10 compared to Group 1. This trend was most apparent on day 10.

**Urinary Metabolites of 2,6-DMA in the Rat**

2,6-DMA was excreted in the urine of treated rats as the parent compound, 4-hydroxy-2,6-DMA and the sulfate and glucoronide conjugates of both of these compounds. N,N,2,6-trimethylamine was detected in the preliminary study, but at concentrations which were too low to allow quantitation (Figure 40 and Table 10).

In rats receiving 2,6-DMA only (Group 5), 4-hydroxy-2,6-DMA was the major excretory product on both Day 1 and 10 of treatment. This
Figure 40. Structures of the excretory products of 2,6-DMA detected in the urine of treated rats.
Table 10. Sum of All Forms (Free, Sulfate Conjugate, Glucuronide Conjugate) of 2,6-DMA Excretory Products Isolated from the Urine of Rats.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Total mg Excreted</th>
<th>Total mM Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,6-DMA</td>
<td>4-OH M/P*</td>
</tr>
<tr>
<td>5(2,6-DMA)</td>
<td>5.58±0.76**</td>
<td>12.76±0.75</td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>3.55±0.41</td>
<td>14.68±0.84</td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>4.21±0.53</td>
<td>25.76±18.62</td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>7.24±1.20</td>
<td>11.89±6.81</td>
</tr>
</tbody>
</table>

Day 5

<table>
<thead>
<tr>
<th></th>
<th>2,6-DMA</th>
<th>4-OH</th>
<th>M/P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5(2,6-DMA)</td>
<td>-+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>2.44±0.38++</td>
<td>15.16±7.03</td>
<td>6.23±2.81</td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>-+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>10.30±4.57</td>
<td>6.96±0.23</td>
<td>0.75±0.24</td>
</tr>
</tbody>
</table>

Day 10

<table>
<thead>
<tr>
<th></th>
<th>2,6-DMA</th>
<th>4-OH</th>
<th>M/P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5(2,6-DMA)</td>
<td>5.18±0.97</td>
<td>14.82±2.23</td>
<td>2.93±0.67</td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>4.00±2.32++</td>
<td>11.41±7.62</td>
<td>2.88±0.76</td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>0.99±0.27</td>
<td>20.69±1.74</td>
<td>22.01±6.41</td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>10.99±4.74</td>
<td>10.99±0.92</td>
<td>1.17±0.55</td>
</tr>
</tbody>
</table>

* Metabolite to parent ratio
** Mean ± Standard deviation
+ Urine was not collected from these treatment groups on day 5.
++ Represents quantity present in the urine of 8, rather than 16, rats.
compound was primarily excreted as the sulfate conjugate, although small amounts of the nonconjugated or glucuronide conjugated forms were isolated on Day 1 and Days 1 and 10, respectively. In contrast, the parent compound was excreted for the most part unconjugated with only a minor degree of sulfate or glucuronide conjugation occurring on either day of treatment. There was no statistical difference on Day 10 as compared to Day 1 in the total excretion of 4-hydroxy-2,6-DMA or the ratio of this metabolite to parent compound.

Animals receiving PB and 2,6-DMA (Group 6) also excreted 4-hydroxy-2,6-DMA as the major urinary product of 2,6-DMA. Sulfate was the predominant conjugating moiety although glucuronidation also occurred. No free hydroxy compound was detected on Day 1 but the nonconjugated form was detected on Day 10. This was in contrast to Group 5 where nonconjugated 4-hydroxy-2,6-DMA was detected on day 1 but not Day 10. Parent compound was primarily excreted without prior conjugation although minor degree of sulfation or glucuronidation was observed. By Day 10 a slightly greater degree of conjugation was found in these animals when compared to their Day 1 values.

Animals in the PB:2,6-DMA group (Group 6) on Day 1 exhibited a significant decrease in excretion as parent compound when compared to Group 5, Day 1. There was no statistically significant change in the total excretion of 4-hydroxy-2,6-DMA or the ratio of metabolite:parent. There was, however, an increase and decrease, respectively, in these values from that found in Group 5 on Day 1, possibly indicating a trend toward greater production of metabolite and decreased excretion as
parent (Table 11a). Direct statistical comparisons between Day 10 values in the PB:2,6-DMA group and other treatment groups are inappropriate owing to the differences in numbers of animals. It would be inappropriate to compare values based on aliquots from the urine of two rats (PB:2,6-DMA on day 10) with that of four rats (all other treatment groups) as results are expressed as total mg or mM excreted. However, an apparent decrease in the amount of 4-hydroxy-2,6-DMA was observed (Table 10). Animals in this group were showing clinical signs on Day 10 similar to those of the PB:2,4-DMA group on day 5. Toxicity could perhaps account for the lower levels of 4-hydroxy-2,6-DMA found in the urine.

Animals in Group 7 (3MC:2,6-DMA) also produced 4-hydroxy-2,6-DMA as the primary excretory product of 2,6-DMA on Days 1 and 10. Sulfate was the predominant conjugating moiety on Day 1; on Day 10 glucuronic acid predominated. Parent compound was excreted primarily in the free form although a small extent of glucuronidation occurred on Days 1 and 10.

Metabolite:parent ratios were significantly increased over those found on Days 1 and 10 in Group 5 by treatment with 3MC (Group 7) (Tables 11a and 11b). Although no significant difference was noted in the total amount excreted as 2,6-DMA or 4-hydroxy-2,6-DMA on Day 1, these values did decrease and increase, respectively, from Group 5 - Day 1 values (Table 11a). Statistically significant differences were detected on day 10 of treatment for both the ratio of metabolite:parent and the amount of 4-hydroxy-2,6-DMA (Table 11b). The amount excreted as 2,6-DMA on Day 10 by Group 7 was 0.99 ± 0.27 mg whereas Group 5 excreted 5.18 ± 0.97 mg
Table 11a. Statistical Comparisons of Groups 6, 7 and 8 Means to Group 5 Means on Day 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total mg Excreted</th>
<th>Total mM Excreted</th>
<th>2,6-DMA Total mg Excreted</th>
<th>2,6-DMA Total mM Excreted</th>
<th>4-Hydroxy Total mg Excreted</th>
<th>4-Hydroxy Total mM Excreted</th>
<th>M/P+ Mg</th>
<th>M/P+ mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5(2,6-DMA)</td>
<td>5.58±0.76</td>
<td>0.05±0.006</td>
<td>12.76±0.75</td>
<td>0.09±0.005</td>
<td>2.32±0.41</td>
<td>2.05±0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>3.55±0.41*</td>
<td>0.03±0.003*</td>
<td>14.68±0.84</td>
<td>0.11±0.006</td>
<td>4.17±0.38</td>
<td>3.68±0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>4.21±0.53</td>
<td>0.03±0.004</td>
<td>25.76±18.62</td>
<td>0.19±0.14</td>
<td>5.89±3.49*</td>
<td>5.19±3.09*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>7.24±1.20*</td>
<td>0.06±0.009*</td>
<td>11.89±6.81</td>
<td>0.08±0.05</td>
<td>1.59±0.76</td>
<td>1.41±0.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11b. Statistical Comparisons of Groups 6, 7 and 8 Means to Group 5 Means on Day 10.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total mg Excreted</th>
<th>Total mM Excreted</th>
<th>2,6-DMA Total mg Excreted</th>
<th>2,6-DMA Total mM Excreted</th>
<th>4-Hydroxy Total mg Excreted</th>
<th>4-Hydroxy Total mM Excreted</th>
<th>M/P Mg</th>
<th>M/P mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5(2,6-DMA)</td>
<td>5.18±0.97</td>
<td>0.04±0.008</td>
<td>14.82±2.23</td>
<td>0.11±0.016</td>
<td>2.93±0.67</td>
<td>2.58±0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>-++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>0.99±0.27</td>
<td>0.008±0.002</td>
<td>20.69±1.74*</td>
<td>0.15±0.012*</td>
<td>22.01±6.41*</td>
<td>19.44±5.67*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>10.99±4.74 *</td>
<td>0.09±0.039*</td>
<td>10.99±0.92*</td>
<td>0.08±0.006*</td>
<td>1.17±0.55</td>
<td>1.04±0.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates a significant difference (p≤0.05).
+ Metabolite to parent ratio (mg or mM Metabolite ÷ mg or mM parent).
++ Values in this group may not be statistically compared due to the difference in numbers of animals producing urine.
Table 11c. Statistical Comparisons of Day 1 vs Day 10 Means for Groups 5, 6, 7 and 8.

<table>
<thead>
<tr>
<th>Group</th>
<th>2,6-DMA (mg)</th>
<th></th>
<th></th>
<th>4-Hydroxy (mg)</th>
<th></th>
<th></th>
<th>M/P (mg)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 vs. Day 10</td>
<td></td>
<td></td>
<td>Day 1 vs. Day 10</td>
<td></td>
<td></td>
<td>Day 1 vs. Day 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5(2,6-DMA)</td>
<td>5.58±0.76</td>
<td>5.18±0.97*</td>
<td>12.76±0.75</td>
<td>14.82±2.23</td>
<td>2.32±0.41</td>
<td>2.93±0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>3.55±0.41</td>
<td>-++</td>
<td>14.68±0.84</td>
<td>-</td>
<td>4.17±0.38</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>4.21±0.53</td>
<td>0.99±0.27</td>
<td>25.76±18.62</td>
<td>20.69±1.74</td>
<td>5.89±3.49</td>
<td>22.01±6.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>7.24±1.20</td>
<td>10.99±4.74</td>
<td>11.89±6.81</td>
<td>10.99±0.92</td>
<td>1.59±0.76</td>
<td>1.17±0.55</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>2,6-DMA (mM)</th>
<th></th>
<th></th>
<th>4-Hydroxy (mM)</th>
<th></th>
<th></th>
<th>M/P (mM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 vs. Day 10</td>
<td></td>
<td></td>
<td>Day 1 vs. Day 10</td>
<td></td>
<td></td>
<td>Day 1 vs. Day 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5(2,6-DMA)</td>
<td>0.05±0.006</td>
<td>0.04±0.008*</td>
<td>0.09±0.005</td>
<td>0.11±0.016</td>
<td>2.05±0.36</td>
<td>2.58±0.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6(PB:2,6-DMA)</td>
<td>0.03±0.003</td>
<td>-</td>
<td>0.11±0.006</td>
<td>-</td>
<td>3.68±0.34</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7(3MC:2,6-DMA)</td>
<td>0.03±0.004</td>
<td>0.008±0.002</td>
<td>0.19±0.14</td>
<td>0.15±0.012</td>
<td>5.19±3.09</td>
<td>19.44±5.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8(SKF:2,6-DMA)</td>
<td>0.06±0.009</td>
<td>0.09±0.039</td>
<td>0.08±0.05</td>
<td>0.08±0.006</td>
<td>1.41±0.67</td>
<td>1.04±0.48</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates a significant difference (p<0.05).
+ Metabolite to parent ratio (mg or mM Metabolite / mg or mM parent).
++ Values in this group may not be statistically compared due to the difference in numbers of animals producing urine.
on Day 10, but this value was not detected as significantly different (Table 11b). Excretion as 2,6-DMA by Group 7 on Day 10 was, however, significantly lower than that found in this group on Day 1 (Table 11c). A corresponding trend towards an increase in the metabolite to parent compound ratio was found (Table 11c).

4-Hydroxy-2,6-DMA was again the major urinary excretory product isolated from animals receiving SKF-525A and 2,6-DMA (Group 8). Sulfate was the predominating conjugating moiety on Days 1, 5, and 10 of treatment. An increase in free 4-hydroxy-2,6-DMA and a decrease in the glucuronide conjugate was observed from days 1 to 10. The parent compound was present in the urine in the free, nonconjugated form.

A significant increase over Group 5 values in the amount excreted as 2,6-DMA was noted on Days 1 and 10 in the SKF-525A group (Table 11a and 11b). This was accompanied by a significant decrease in 4-hydroxy-2,6-DMA on Day 10 compared to Group 5's Day 10 value. No difference from Group 5 in the metabolite:parent ratio was detected on either Day 1 or Day 10, but the mean values tended to be lower than those observed in Group 5 (Table 11a and 11b). There was no difference in the amount excreted as parent, 4-hydroxy-2,6-DMA or the metabolite:parent ratio on Day 10 as compared to Day 1 (Table 11c).

In summary, the metabolic pathway of 2,6-DMA was not altered by 1 or 10 days of treatment or by concurrent treatment with PB, 3MC, or SKF-525A. 4-Hydroxy-2,6-DMA was the major excretory product in all groups and on all days of treatment. This compound was excreted by all groups
primarily as the sulfate conjugate with only one exception; 3MC treatment for 10 days increased glucuronidation over sulfation. The parent compound was detected in urine in greatest amounts without prior conjugation.

Differences in the quantities of excretory products due to the length of treatment or concurrent administration of PB, 3MC, or SKF-525A were observed. PB treatment tended to increase the excretion of 4-hydroxy-2,6-DMA and decrease the excretion of 2,6-DMA. The same trend was noted with 3MC, but the change here was more marked. In contrast, SKF-525A enhanced the excretion of unchanged parent compound at the expense of 4-hydroxy-2,6-DMA.

Urinary Metabolites of 2,4-DMA in the Dog

2,4-DMA was excreted in the urine of treated dogs as the parent compound, 6-hydroxy-2,4-DMA, 4-amino-3-methyl-benzoic acid and the sulfate and glucuronide conjugates of these compounds. N,N,2,4-trimethylaniline and the glycine conjugate of 4-amino-3-methyl-benzoic acid were also detected in the preliminary study, but at concentrations which were too low to allow quantitation (Figure 41).

The major excretory product of 2,4-DMA in dog urine on both Day 1 and Day 10 of treatment was 6-hydroxy-2,4-DMA. The metabolite was primarily excreted without prior conjugation, although glucuronidation did occur and the sulfate conjugate was detected in one animal. Conjugation with
Figure 41. Structures of the excretory products of 2,4-DMA detected in the urine of treated dogs.
glucuronic acid was highly variable. Certain dogs produced the conjugated product at levels roughly equal to the free form, while others produced little or no conjugate. Excretion of the hydroxylated metabolite on Day 10 was also variable. Both increases and decreases in the quantity excreted were observed for individual dogs, when compared to those obtained on Day 1. The metabolite was excreted both in the free form and as the glucuronide conjugate; evidence for sulfation was found in only one sample. There was no statistical difference between Days 1 and 10 in the total excretion of the hydroxylated metabolite or its metabolite to parent ratio (Table 12).

The parent compound was excreted on Day 1 as the free form and as the glucuronide conjugate in roughly equal quantities. No evidence for sulfation was observed. By Day 10, the quantity conjugated with glucuronic acid had dropped and a degree of sulfation was found. There was no significant difference between Days 1 and 10 in the total excretion as parent compound (Table 12).

The compound 4-amino-3-methyl-benzoic acid was present in the urine on both Days 1 and 10 as a minor metabolite. The compound was excreted in the free form and as the sulfate and glucuronide conjugates. The glucuronide conjugate was the predominant urinary form on Day 1, whereas on Day 10 the free metabolite was present in greater quantities. No significant difference was detected between Days 1 and 10 in the total amount of 4-amino-3-methyl-benzoic acid excreted or its ratio to parent compound (Table 12).
Table 12. Sum of all Forms (Free, Sulfate Conjugate, Glucuronide Conjugate) of 2,4-DMA Excretory Products Isolated from the Urine of Dogs.

Day 1

<table>
<thead>
<tr>
<th>Dog #</th>
<th>2,4-DMA Total mg Excreted</th>
<th>6-OH Total mg Excreted</th>
<th>M/P+ Total mg Excreted</th>
<th>4-COOH Total mg Excreted</th>
<th>M/P+ Total mg Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM+</td>
<td>mg</td>
<td>mM</td>
<td>mg</td>
<td>mM</td>
</tr>
<tr>
<td>1</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.28 0.027</td>
<td>28.08 0.205</td>
<td>8.56 7.56</td>
<td>1.68 0.011</td>
<td>0.512 0.410</td>
</tr>
<tr>
<td>3</td>
<td>10.20 0.084</td>
<td>111.90 0.817</td>
<td>10.97 9.69</td>
<td>2.92 0.019</td>
<td>0.286 0.229</td>
</tr>
<tr>
<td>4</td>
<td>5.78 0.048</td>
<td>129.02 0.942</td>
<td>22.32 19.71</td>
<td>1.97 0.013</td>
<td>0.341 0.273</td>
</tr>
<tr>
<td>5</td>
<td>15.80 0.131</td>
<td>161.42 1.18</td>
<td>10.22 9.02</td>
<td>3.87 0.026</td>
<td>0.245 0.196</td>
</tr>
<tr>
<td>Mean</td>
<td>8.77 0.072</td>
<td>107.61 0.79</td>
<td>13.02 11.49</td>
<td>2.61 0.017</td>
<td>0.346 0.277</td>
</tr>
<tr>
<td>Stand. Dev.</td>
<td>5.49 0.045</td>
<td>56.85 0.41</td>
<td>6.28 5.55</td>
<td>0.99 0.006</td>
<td>0.118 0.094</td>
</tr>
</tbody>
</table>

Day 10

<table>
<thead>
<tr>
<th>Dog #</th>
<th>2,4-DMA Total mg Excreted</th>
<th>6-OH Total mg Excreted</th>
<th>M/P+ Total mg Excreted</th>
<th>4-COOH Total mg Excreted</th>
<th>M/P+ Total mg Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM+</td>
<td>mg</td>
<td>mM</td>
<td>mg</td>
<td>mM</td>
</tr>
<tr>
<td>1</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18.71 0.155</td>
<td>116.39 0.849</td>
<td>6.22 5.49</td>
<td>4.36 0.029</td>
<td>0.233 0.187</td>
</tr>
<tr>
<td>3</td>
<td>0.61 0.005</td>
<td>11.16 0.081</td>
<td>18.29 16.16</td>
<td>0.43 0.003</td>
<td>0.705 0.564</td>
</tr>
<tr>
<td>4</td>
<td>5.06 0.042</td>
<td>134.25 0.979</td>
<td>26.53 23.43</td>
<td>1.27 0.008</td>
<td>0.251 0.201</td>
</tr>
<tr>
<td>5</td>
<td>13.63 0.113</td>
<td>26.93 0.197</td>
<td>1.98 1.75</td>
<td>0.63 0.004</td>
<td>0.046 0.037</td>
</tr>
<tr>
<td>Mean</td>
<td>9.50 0.078</td>
<td>72.18 0.527</td>
<td>13.26 11.71</td>
<td>1.67 0.011</td>
<td>0.309 0.247</td>
</tr>
<tr>
<td>Stand. Dev.</td>
<td>8.18 0.067</td>
<td>62.12 0.453</td>
<td>11.23 9.92</td>
<td>1.83 0.012</td>
<td>0.279 0.224</td>
</tr>
</tbody>
</table>

+ Ratio of metabolite to parent (mg or mM metabolite / mg or mM parent).
++ Total mg excreted expressed as millimoles (mM)
* Dog vomited shortly after dosing.
** No peaks were found on the chromatogram of this sample.
In summary, the major metabolic pathways of 2,4-DMA were not altered by 1 or 10 days of treatment. Considerable variation between individual dogs was found. The variability may be related to some quality inherent to 2,4-DMA and its metabolites or to some aspect of dog urine and its collection. The results of the 2,4-DMA rat urine analysis were more variable than that of 2,6-DMA urine analysis. The overall variability of the analysis of dog urine was greater than that seen in rats.

Urinary Metabolites of 2,6-DMA in the Dog

2,6-DMA was excreted in the urine of treated dogs as the parent compound, 4-hydroxy-2,6-DMA, 2-amino-3-methyl-benzoic acid and as the sulfate and glucuronide conjugates of these compounds. An unknown metabolite, molecular weight 135 and tentatively identified as 4-imino-3,5-dimethyl-quinone, was also detected. N,2,6-trimethylaniline, 2,6-dimethyl-nitrosobenzene and the glycine conjugate of 2-amino-3-methyl-benzoic acid were detected in the preliminary study, but at levels which were too low to allow quantification (Figure 42).

The major excretory product of 2,6-DMA detected in dog urine after 1 or 10 days of treatment was 4-hydroxy-2,6-DMA. On Day 1, the metabolite was excreted in roughly equal amounts as the sulfate or glucuronide conjugates. No free hydroxylates product was isolated. By Day 10 the extent of glucuronidation had decreased and the sulfate conjugate was the predominant form. As on Day 1, no free hydroxy compound was
Figure 42. Structures of the excretory products of 2,6-DMA detected in the urine of treated dogs.
isolated in the urine. There was no statistical difference in the total excretion of 4-hydroxy-2,6-DMA on Day 1 vs. Day 10 (Table 13).

Parent compound was excreted on Day 1 as the free compound, the sulfate and the glucuronide conjugates in roughly equal quantities. On Day 10, levels of free 2,6-DMA had increased over those measured on Day 1. There was no recognizable trend in the sulfation or glucuronidation of the parent compound on Day 10. No significant difference was detected in the amount excreted as parent compound on Day 10 compared to Day 1 (Table 13).

The compound, 2-amino-3-methyl-benzoic acid, was detected in the urine of treated dogs as a minor metabolite. On Day 1, the metabolite was excreted in approximately equal quantities as the free compound or conjugated to glucuronic acid. Sulfate conjugation was variable. By Day 10, a greater degree of conjugation was seen than that observed on Day 1. Total excretion as the benzoic acid derivative had decreased significantly from that observed on Day 1 (Table 13).

In summary, the major metabolic pathways of 2,6-DMA were not altered by 1 or 10 days of treatment. Length of treatment did however, significantly affect the urinary level of the minor metabolite, 2-amino-3-methyl-benzoic acid. No significant difference was detected in the amount excreted as parent compound or 4-hydroxy-2,6-DMA on Day 10 compared to Day 1, but a possible trend toward decreasing amounts of these compounds was observed on Day 10. A decrease in the total
Table 13. Sum of all Forms (Free, Sulfate Conjugate, Glycuronide Conjugate) of 2,6-DMA Excretory Products Isolated from the Urine of Dogs.

Day 1

<table>
<thead>
<tr>
<th>Dog #</th>
<th>2,6-DMA</th>
<th>4-OH</th>
<th>M/P+</th>
<th>2-COOH</th>
<th>M/P+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total mg Excreted</td>
<td>mM+</td>
<td>Total mg Excreted</td>
<td>mM</td>
<td>mg</td>
</tr>
<tr>
<td>1</td>
<td>27.62</td>
<td>0.228</td>
<td>183.15</td>
<td>1.337</td>
<td>6.63</td>
</tr>
<tr>
<td>2</td>
<td>17.00</td>
<td>0.140</td>
<td>246.13</td>
<td>1.797</td>
<td>14.47</td>
</tr>
<tr>
<td>3</td>
<td>5.07</td>
<td>0.042</td>
<td>43.99</td>
<td>0.321</td>
<td>8.67</td>
</tr>
<tr>
<td>4</td>
<td>20.45</td>
<td>0.169</td>
<td>59.74</td>
<td>0.436</td>
<td>2.92</td>
</tr>
<tr>
<td>5</td>
<td>6.30</td>
<td>0.052</td>
<td>57.82</td>
<td>0.422</td>
<td>9.17</td>
</tr>
<tr>
<td>Mean</td>
<td>15.29</td>
<td>0.126</td>
<td>118.17</td>
<td>0.863</td>
<td>8.37</td>
</tr>
<tr>
<td>Stand.Dev.</td>
<td>9.58</td>
<td>0.079</td>
<td>91.04</td>
<td>0.665</td>
<td>4.20</td>
</tr>
</tbody>
</table>

Day 10

<table>
<thead>
<tr>
<th>Dog #</th>
<th>2,6-DMA</th>
<th>4-OH</th>
<th>M/P+</th>
<th>2-COOH</th>
<th>M/P+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total mg Excreted</td>
<td>mM+</td>
<td>Total mg Excreted</td>
<td>mM</td>
<td>mg</td>
</tr>
<tr>
<td>1</td>
<td>4.36</td>
<td>0.036</td>
<td>32.96</td>
<td>0.241</td>
<td>7.56</td>
</tr>
<tr>
<td>2</td>
<td>12.94</td>
<td>0.107</td>
<td>6.32</td>
<td>0.046</td>
<td>0.49</td>
</tr>
<tr>
<td>3</td>
<td>9.37</td>
<td>0.077</td>
<td>25.36</td>
<td>0.185</td>
<td>2.71</td>
</tr>
<tr>
<td>4</td>
<td>18.32</td>
<td>0.151</td>
<td>32.80</td>
<td>0.239</td>
<td>1.79</td>
</tr>
<tr>
<td>5</td>
<td>11.50</td>
<td>0.095</td>
<td>63.13</td>
<td>0.461</td>
<td>5.45</td>
</tr>
<tr>
<td>Mean</td>
<td>11.29</td>
<td>0.093</td>
<td>32.11</td>
<td>0.234</td>
<td>3.61</td>
</tr>
<tr>
<td>Stand.Dev.</td>
<td>5.09</td>
<td>0.042</td>
<td>20.46</td>
<td>0.149</td>
<td>2.87</td>
</tr>
</tbody>
</table>

* Significantly different (p≤0.05) from day 1.
+ Ratio of metabolite to parent (mg or mM metabolite ÷ mg or mM parent)
++ Total mg excreted expressed as millimoles (mM)
elimination of 2,6-DMA could be consistent with the induction of hepatotoxicity.

Covalent Binding

Significant differences were observed in the covalent binding of \(^{14}\text{C}\)-2,6-DMA and \(^{14}\text{C}\)-2,4-DMA to hepatic DNA, RNA and protein (Table 14). Differences were also observed in hepatic RNA and protein concentrations between animals treated with 2,4-DMA and those in the control or 2,6-DMA treatment groups (Table 15).

The covalent binding index (CBI) and picomoles bound/mg DNA, RNA or protein were significantly higher in animals treated with \(^{14}\text{C}\)-2,4-DMA compared to animals receiving \(^{14}\text{C}\)-2,6-DMA. The mean CBI for \(^{14}\text{C}\)-2,4-DMA was 21.73 ± 9.82 vs 7.85 ± 6.95 for the \(^{14}\text{C}\)-2,6-DMA. Likewise, the binding of \(^{14}\text{C}\)-2,4-DMA to DNA on a picomole/mg basis was significantly higher than that of \(^{14}\text{C}\)-2,6-DMA, 2.11 ± 0.95 and 0.75 ± 0.65, respectively. Covalent binding to RNA and protein was also significantly greater in animals treated with \(^{14}\text{C}\)-2,4-dMA compared to \(^{14}\text{C}\)-2,6-DMA. \(^{14}\text{C}\)-2,4-DMA binding to hepatic RNA and protein was 9.60 ± 2.04 pM/mg and 21.93 ± 2.69 pM/mg, respectively. Values of 1.82 ± 0.87 pM/mg and 7.00 ± 1.88 pM/mg were obtained for the binding of \(^{14}\text{C}\)-2,6-DMA to hepatic RNA and protein, respectively. Mean RNA protein content was 1.36 ± 0.25 mg for animals treated with 2,6-DMA and 0.02 ± 0.05 mg for those treated with 2,4-DMA.
Table 14. Covalent Binding of $[^{14}\text{C}]-2,4$-DMA and $[^{14}\text{C}]-2,6$-DMA to Liver DNA, RNA and Protein Following a 9 Day Pretreatment with Cold Compound.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CBI (pm Bound/mg DNA)</th>
<th>pM Bound/mg DNA</th>
<th>pM Bound/mg RNA</th>
<th>pM Bound/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{14}\text{C}]-2,4$-DMA</td>
<td>$21.73\pm 9.82^*\pm 7.85\pm 6.95$</td>
<td>$2.11\pm 0.95^*$</td>
<td>$0.75\pm 0.65$</td>
<td>$9.60\pm 2.04^*\pm 1.82\pm 0.87$</td>
</tr>
<tr>
<td>$[^{14}\text{C}]-2,6$-DMA</td>
<td>$7.85\pm 6.95$</td>
<td>$21.93\pm 2.69^*$</td>
<td>$7.00\pm 1.88$</td>
<td></td>
</tr>
</tbody>
</table>

+ Mean ± standard deviation, n = 6.
* Significantly different (p≤0.05) from $[^{14}\text{C}]-2,6$-DMA.

Table 15. Comparison of the Hepatic DNA, RNA and Protein Concentrations in Control, $[^{14}\text{C}]-2,4$-DMA, and $[^{14}\text{C}]-2,6$-DMA Animals.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DNA (mg/g liver)</th>
<th>RNA (mg/g liver)</th>
<th>Protein (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 2,4-DMA</td>
<td>Control 2,4-DMA</td>
<td>Control 2,4-DMA</td>
</tr>
<tr>
<td></td>
<td>2,6-DMA</td>
<td>2,6-DMA</td>
<td>2,6-DMA</td>
</tr>
<tr>
<td>DNA (mg/g liver)</td>
<td>Control 2,4-DMA</td>
<td>Control 2,4-DMA</td>
<td>Control 2,4-DMA</td>
</tr>
<tr>
<td></td>
<td>2,6-DMA</td>
<td>2,6-DMA</td>
<td>2,6-DMA</td>
</tr>
<tr>
<td></td>
<td>0.66±0.08^</td>
<td>0.50±0.28</td>
<td>0.61±0.14</td>
</tr>
<tr>
<td></td>
<td>5.01±0.77</td>
<td>1.76±0.73^</td>
<td>5.22±1.22</td>
</tr>
<tr>
<td></td>
<td>59.86±9.16</td>
<td>89.27±7.92^</td>
<td>66.44±12.77</td>
</tr>
</tbody>
</table>

+ Mean ± standard deviation, n = 6.
* Significantly different (p≤0.05) from control values.
A 10 day treatment with 2,4-DMA decreased RNA concentrations compared to control and 2,6-DMA animals (Table 15). Protein concentrations were increased by 2,4-DMA in contrast to the effect on RNA concentrations. No effect was found on the concentration of DNA. 2,6-DMA, however, did not change hepatic DNA, RNA or protein concentrations from that of the controls (Table 15).
DISCUSSION

The primary goal of this study was to determine whether or not the species specific hepatic damage caused by 2,4-DMA and 2,6-DMA is related to different patterns of metabolism. This is a logical hypothesis based on what is known about species differences in biotransformation and the metabolic activation of xenobiotics to reactive species. Species differences in the metabolism of drugs and other xenobiotics is a well known phenomenon. Examples of a species effect are found in the metabolism of acetaminophen in the dog and cat, of ephedrine in the dog, rat, guinea pig and rabbit, of aniline in the dog and rat, and of MDA in the rat and rabbit. The metabolic activation of chemicals to potent alkylating, arylating or acylating agents has been implicated in the toxicity, mutagenesis and carcinogenicity of various xenobiotics. Activation to chemically reactive metabolites has been shown to be involved with the toxicity of acetaminophen, the mutagenesis of aflatoxin B1 and the carcinogenicity of 2-acetylaminofluorene as well as the toxic effects of many other compounds. Additionally, the liver is inherently susceptible to the toxic effects of metabolically activated compounds due to its central role in drug metabolism and the high concentrations of ingested compounds it receives via the portal circulation.

Identification of the metabolites of 2,4-DMA and 2,6-DMA in the rat and
the dog was required to evaluate the effects of biotransformation on hepatic lesions. No reports on the metabolism of 2,4- and 2,6-DMA in the dog were found in the literature, and although the metabolites of the two isomers had been investigated in the rat, a more definitive study was needed. The results of the present study indicated that the rat and dog exhibited a significant species specific effect in the metabolism of the two isomers. The dog was also apparently capable of metabolizing the two isomers to a greater variety of products than the rat. Substantial differences in the metabolism of 2,4- and 2,6-DMA by the rat were also found.

Although the results of this study corresponded with the previously reported hepatotoxic effects of 2,6-DMA in the rat and the dog and with that reported for 2,4-DMA in the dog, certain differences were found in the urinary metabolites of 2,4- and 2,6-DMA and the hepatic pathology induced by 2,4-DMA in the rat. In order to more easily relate the above findings, this discussion is organized into three sections: the hepatotoxicity of 2,4- and 2,6-DMA in the rat and dog, the urinary metabolites of the two isomers in the two species and the covalent binding of $[^{14}\text{C}]-2,4$-DMA and $[^{14}\text{C}]-2,6$-DMA to hepatic macromolecules in the rat.

**Hepatotoxicity**

2,4-DMA has been reported to cause a greater increase in the liver weight of treated rats than 2,6-DMA.15,45,46,47 This effect was substantiated in the present study where 2,4-DMA, administered alone or
in conjunction with PB, 3MC, or SKF-525A, consistently increased liver weights over corresponding control and 2,6-DMA animals. Liver to body weight ratios were also increased in the above 2,4-DMA treatment groups. Enzymatic modification by PB, 3MC, or SKF-525A did not alter the ability of 2,4-DMA to increase liver weight or the liver to body weight ratio, as 2,4-DMA treatment consistently increased these values over those of corresponding control groups. In contrast, 2,6-DMA did not alter liver weight or the liver to body weight ratio from control values when administered alone or in conjunction with PB, 3MC, or SKF-525A. These findings were consistent with earlier reports\(^{15,45,46,47}\) on the effects of the two isomers on rat liver weights and, in addition, demonstrated that PB, 3MC, or SKF-525A treatment did not alter these effects.

In contrast to the effects on liver weight and liver to body weight ratios, total body weight was not altered from corresponding control values by treatment with 2,4-DMA alone or in conjunction with PB, 3MC or SKF-525A. 2,6-DMA, administered alone or with 3MC or SKF-525A, also had no effect on body weight. However, the combination of PB and 2,6-DMA induced a significant decrease in body weight compared to PB control animals or those treated with 2,6-DMA alone. This effect was observed on both Day 5 and Day 10 of treatment.

The decrease in body weight in the PB:2,6-DMA treatment groups appeared to indicate some toxic synergistic effect between PB and 2,6-DMA. In addition to weight loss, these animals also exhibited clinical symptoms of distress (depression, roughened hair coat, chromodacryorrhea) by Day
of treatment. A hepatic lesion distinct to this treatment group was not observed at necropsy or on light or electron microscopic evaluation. An isolated zone of hepatic necrosis was observed via light microscopy in several animals of the PB:2,6-DMA group, but as this change was also seen in the PB:Control and PB:2,4-DMA groups it would be incorrect to attribute this lesion solely to treatment with PB and 2,6-DMA.

The fact that the combination of PB and a dialkylaniline was able to cause a toxic effect was substantiated by the loss of 50% of the PB:2,4-DMA treatment group by Day 5. Similar to the weight loss in the PB:2,6-DMA groups, no direct cause of death was found at necropsy or on light or electron microscopic evaluation. However, both the weight loss in the PB:2,6-DMA animals and the mortality in the PB:2,4-DMA group may have involved some interference in the elimination of PB or the dialkylaniline isomers. Animals in the PB:2,4-DMA group were observed to be in a moribund condition prior to death. As it is impossible on clinical observation to distinguish between a prolonged or enhanced effect of PB and an animal unconscious due to a toxic effect, these animals could have been suffering from an alteration in the clearance of PB. Alternately, the clearance of 2,4-DMA could have been affected by PB. The same inference may be applied to the weight loss and clinical signs observed in the PB:2,6-DMA treatment groups, signs of toxicity taking longer to develop and being less severe in nature in these animals due to the lesser effect of 2,6-DMA, as compared to 2,4-DMA, in the rat.

Mention of the effects of PB, 3MC and SKF-525A in corn oil gavaged
controls should be made as the duration of treatment with these compounds was somewhat unusual. Body weights were decreased from rats receiving corn oil alone by 10 days of treatment with 3MC or SKF-525A at 15 mg/kg or 50 mg/kg, respectively. PB, at 80 mg/kg for the same duration of treatment, had no such effect. Liver weights were increased by 3MC and PB, but not by SKF-525A. The liver to body weight ratio, however, was increased by PB, 3MC and SKF-525A. An apparent toxicity was produced by 10 days of treatment with 3MC or SKF-525A as evidenced by the decrease in body weight.

An increase in liver weight in response to PB or 3MC treatment is a common response and is thought to be associated with their capacity for enzyme induction. The exact relationship between enzyme induction and an increase in liver weight is unclear as enzyme induction can take place without enlargement of the liver. The opposite situation, i.e. an increase in liver weight (or size) without enzyme induction, may occur as well. The mechanisms by which PB and 3MC produce enzyme induction are different, as are the spectra of drug metabolizing enzymes induced and the associated morphologic liver changes. 3MC appears to bind to a cytosolic receptor and is transported to the nucleus where it interacts with structural genes to stimulate enzyme induction; no specific PB receptor has been found and its mechanism of induction is unclear. 3MC has been termed a "specific inducer", stimulating a specific isoenzyme of cytochrome P-450 (P-448 or P-450c) whereas PB is a more "general inducer" stimulating P-450b and thus the metabolism of a wider range of chemicals. PB also significantly increases the SER content of hepatocytes, while 3MC
little effect in this regard. Because of these differences in enzymatic patterns, mechanisms of induction and morphologic changes produced by PB and 3MC, it is probable that their causes of liver enlargement are different as well.

SKF-525A, an inhibitor of microsomal oxidation, produced no statistical change in liver weight from corn oil gavaged control animals. The increase in liver to body weight ratio observed in SKF-525A - treated control animals was probably related to the SKF-525A induced decrease in body weight and not to any effect on the liver. 3MC treatment also decreased body weight, but at the same time produced an increase in liver weight and thus a change in the liver to body weight ratio. PB, on the other hand, increased liver weight but had no effect on body weight.

A direct comparison between the effects of 2,4- and 2,6-DMA on the liver weights of dogs and rats was not possible as dogs were not sacrificed at the end of the dosing period. However, the livers of 2,6-DMA treated dogs appeared enlarged and swollen on visual observation during biopsy on day 11, whereas those in the 2,4-DMA treatment group appeared normal in size. Furthermore, a greater body weight loss, 2.38 + 0.48 kg vs. 0.68 + 0.40 kg, was found in dogs treated with 2,6-DMA as opposed to 2,4-DMA, respectively. These findings were consistent with the greater toxicity of 2,6-DMA compared to 2,4-DMA in the dog and were comparable to results reported previously.

With regard to the histopathologic effect of 2,4-DMA (Group 1) in the
rat, the present study indicated hepatocyte enlargement, a loss of sinusoids and segregation or clumping or subcellular organelles. The results of this study may be compared to those of Short et al. as the dose and duration of treatment were identical. Short et al. reported cloudy swelling, diffuse hepatocellular necrosis, early periacinar connective tissue proliferation, biliary hyperplasia and periacinar vacuolar degeneration. In the present study, no connective tissue proliferation, necrosis or biliary hyperplasia were observed. Scattered lipid vacuoles were found on electron microscopy. The lesion described as cloudy swelling in the Short study may have corresponded to the cytoplasmic change observed in rats treated with 2,4-DMA in the present study. This cytoplasmic change was distinct but subtle and difficult to describe. The pathologic definition of cloudy swelling is inexact, and it is possible that this term was used to describe a lesion similar to that found in the present study. The absence of biliary hyperplasia is more difficult to resolve. It is possible that individual variation is important in the development of biliary hyperplasia with low doses of 2,4-DMA and that high doses are required to produce this effect consistently. It is of interest to note that Magnusson et al. reported a slight proliferation of bile ducts in rats treated with 2,4-DMA at 500-700 mg/kg/day for four weeks, but not in those receiving 20 or 100 mg/kg/day for the same duration of time.46 Grasso, et al. also noted no histological changes in the livers of rats gavaged with 2,4-DMA at 10, 25, 50, 100 or 250 mg/kg for 7 days.123 Furthermore, focal hepatic necrosis and vacuolization were noted only in the high dose treatment group in Magnusson's study.46
No comparable studies of the effects of PB, 3MC or SKF-525A given in conjunction with 2,4-DMA on liver morphology were available in the literature. Subsequently no historical comparisons can be made, but a comparison of the effects of these enzyme modifiers on 2,4-DMA pathology is appropriate. The histologic appearance of livers from animals treated with PB and 2,4-DMA (Day 5) or 3MC and 2,4-DMA (Day 10) closely resembled that of animals receiving 2,4-DMA alone on Days 5 and 10, respectively. However, the cytoplasmic change produced by 2,4-DMA was more pronounced in the 3MC:2,4-DMA treatment group than in animals treated with 2,4-DMA alone or 2,4-DMA in conjunction with PB. Based on these results and those of the organ weight analysis, it would appear that 3MC had a greater ability than PB to effect the specific changes produced by 2,4-DMA. The possibility that treatment with PB and 2,4-DMA may have induced a change different from that of 2,4-DMA, had any of these animals survived to Day 10, cannot be ignored.

The effect of 2,4-DMA in combination with SKF-525A on liver morphology requires prior reference to the hepatic effect of SKF-525A. SKF-525A at 50 mg/kg/day for 10 days produced a midzonal fatty degeneration in the livers of treated rats. No study of comparable duration describing the histopathologic effect of SKF-525A on the liver was found in the literature, but the compound has been used in numerous short term studies to evaluate the relationship between toxicity and metabolism. Its usefulness in such studies is due to its ability to inhibit mixed function oxidase activity and thus aid in the differentiation between direct acting toxicants and those which require metabolic activation. However, inhibition of the microsomal
mixed function oxidase is not the only effect of SKF-525A. Its actions within the cell are many and varied. SKF-525A has been found to inhibit the membrane-transport of amino acids, the incorporation of amino acids into proteins, the synthesis of cholesterol and the reabsorption of sodium and chloride by the kidney. An effect on the osmotic and excitable properties of cell membranes, a decrease in hepatic glycogen and glutathione and an inhibition of mitochondrial respiration has also been observed. Any one or a combination of the above effects of SKF-525A could have contributed to the production of the midzonal fatty degeneration detected in this study. The production of a midzonal lesion is somewhat unusual, most hepatotoxicants acting preferentially in the centrilobular or periportal regions. Oxygen, nutrient and enzyme gradients are known to exist across the hepatic lobule. The midzonal region has been less well studied in this regard than the centrilobular or periportal zones.

At the present time, the particular attributes of SKF-525A and hepatic midzonal cells which led to the production of the fatty degeneration remain to be elucidated. However, several causes may be proposed. Firstly, SKF-525A has been shown to inhibit mitochondrial respiration in vitro. Beta-oxidation of fatty acids by the mitochondria is an energy requiring process and is one of two major pathways for the handling of lipids by the hepatocyte. A decrease in the production of energy by an inhibition of respiration could, therefore, interfere with the beta-oxidation of fatty acids. Impaired beta-oxidation may subsequently allow the accumulation of lipid within the hepatocyte. The production of ethanol-induced fatty liver in man
has been associated with an impairment in mitochondrial oxidation.\textsuperscript{128} Alternately, the ability of SKF-525A to inhibit the incorporation of amino acids into protein and cholesterol synthesis could have affected the normal processing of fatty acids within the hepatocytes. The hepatic incorporation of fatty acids into complex lipids, such as triglycerides or cholesterol esters is a second major pathway of lipid metabolism.\textsuperscript{128} Once synthesized, the complex lipids may be secreted into the blood or used for the production of cellular membranes. Secretion of complex lipids from the liver requires the coupling of lipid to protein. Inhibition of the synthesis of protein or cholesterol by SKF-525A could therefore have blocked this pathway at some point. Finally, SKF-525A has been shown to deplete hepatic glutathione in a strain and species dependent manner.\textsuperscript{125} Glutathione performs many important functions within the cell; it maintains the sulfhydryl groups of protein, aids in the translocation of amino acids, acts as a scavenger of hydrogen peroxide, lipid peroxides and free radicals produced during normal metabolism, and aids in the detoxification of foreign compounds.\textsuperscript{129} Changes in hepatocellular glutathione levels, such as that induced by SKF-525A, could therefore alter normal cellular integrity and function. Such an effect was found in Fischer 344 rats following a single treatment with SKF-525A at 100 mg/kg, where depressed hepatic glutathione levels were accompanied by a rise in SGPT concentrations.\textsuperscript{125} SGPT is a serum enzyme used clinically in the detection of liver diseases and elevated concentrations are thought to indicate a change in cell membrane permeability due to injury or death. Therefore, an apparent hepatotoxic effect was produced by a single dose of SKF-525A and a 10 day treatment,
as was used in the present study, could potentially exacerbate such an effect. The accumulation of lipid, in response to a hepatotoxic chemical, is not uncommon.\textsuperscript{126,128}

In contrast to those receiving SKF-525A alone, rats treated with 2,4-DMA in conjunction with SKF-525A exhibited no (or minimal) lipid accumulation. Furthermore, the characteristic cytoplasmic appearance typical of treatment with 2,4-DMA was not observed in this group. The increase in liver weight and liver to body weight ratio, which was also typical of 2,4-DMA treatment, was observed in rats receiving SKF-525A in conjunction with 2,4-DMA. Apparently some, but not all, of the effects of 2,4-DMA were alleviated by concurrent treatment with SKF-525A. Likewise, treatment with 2,4-DMA was able to alter some aspect(s) of SKF-525A toxicity as evidenced by the alleviation of the midzonal fatty degeneration. Animals in the SKF:2,6-DMA treatment group exhibited a decrease in the severity of the midzonal fatty degeneration when compared to animals receiving SKF-525A alone. Therefore, 2,6-DMA appeared less effective in reducing the midzonal lipid accumulation due to SKF-525A treatment than 2,4-DMA.

The occurrence of midzonal fatty degeneration in SKF-525A control animals and the ability of 2,4-DMA to minimize, and of 2,6-DMA to lessen, this change were unexpected findings. However, the ability of one xenobiotic to alter the absorption, distribution, metabolism or excretion of another is a well known phenomenon. It was because of this phenomenon that the enzyme modifiers PB, 3MC and SKF-525A were included in this study. 2,4-DMA, and to a lesser extent 2,6-DMA, have been shown to be
capable of inducing drug metabolizing enzyme activity and altering certain biochemical parameters within the cell. These capabilities may have been important in the effects of the two isomers on the hepatotoxicity of SKF-525A. It is interesting to note that in a study where the hepatic effects of SKF-525A were investigated, 3MC pretreatment eliminated the SKF-525A - induced glutathione depletion in mice. The authors concluded that the protectant effect of 3MC was due to an alteration in the oxidative metabolism of SKF-525A. In the present study, 3MC treatment enhanced the histopathologic and electron microscopic changes induced by 2,4-DMA. This would appear to indicate that 2,4-DMA was metabolized via pathways affected by 3MC. 2,4-DMA appears to have enzyme inductive effects of its own, as reflected by increases in cytochrome P-450 and microsomal protein. If the pathways induced by 3MC and 2,4-DMA are similar and if the SKF-525A - induced lesion is related to an activated product of SKF-525A, an alteration in the biotransformation of SKF-525A by 2,4-DMA may have been responsible for the substantial decrease in midzonal fatty degeneration in these animals. Using the same reasoning, 2,6-DMA would not be expected to have as great an effect as 2,4-DMA. 2,6-DMA is a less effective inducer than 2,4-DMA and concurrent treatment with 3MC and 2,6-DMA in the present study was without significant effect.

With respect to the effect of 2,6-DMA on the livers of rats, the lack of a hepatotoxic effect of 2,6-DMA (Group 5) observed in this study was consistent with that reported previously. Liver sections from rats treated with 2,6-DMA, PB and 2,6-DMA or 3MC and 2,6-DMA were similar in histologic appearance to corresponding control groups.
Treatment with 3MC and 2,6-DMA appeared to slightly increase the lipid vacuolization observed in 3MC animals. These findings corresponded with the failure of 2,6-DMA, alone or in conjunction with PB or 3MC, to alter liver weight or liver to body weight ratios as compared to corresponding control groups. PB and 3MC, therefore, appeared unable to alter 2,6-DMA metabolism in a manner which significantly affected hepatic morphology. The effect of SKF-525A and 2,6-DMA has been discussed previously.

The histopathologic effects of 2,4- and 2,6-DMA on the livers of treated dogs were also compatible with those reported previously. Oral treatment with 2,6-DMA at 2, 10 or 50 mg/kg/day for 4 weeks produced hepatic fatty degeneration at all dose levels, while a mild degree of fatty degeneration was produced by 2,4-DMA at the high dose only. In the present study, the production of centrilobular fatty degeneration by 2,6-DMA and the lack of effect by 2,4-DMA were consistent findings throughout the treatment groups. Dose and duration of treatment were 25 mg/kg/day and 10 days, respectively. Therefore, 2,6-DMA is capable of producing its hepatotoxic effect with a shorter duration of treatment than has been reported previously.

**Urinary Metabolites**

As stated at the beginning of this discussion, certain differences were found between the urinary metabolites of 2,4- and 2,6-DMA in the rat detected in the present study and those reported previously. Lindstrom reported the presence of N-acetyl-2,4-DMA and 4-amino-3-methyl-benzoic acid and its glycine conjugate in the urine of rats treated with
2,4-DMA,\textsuperscript{44} and 2-amino-3-methyl-benzoic acid in the urine of rats treated with 2,6-DMA.\textsuperscript{45} These compounds were not detected in the present study, although the methods employed were capable of their isolation and detection. The chemical standard of N-acetyl-2,4-DMA was readily detected in the preliminary study. 4-Amino-3-methyl-benzoic acid, its glycine conjugate and 2-amino-3-methyl-benzoic acid were detected in the urine of dogs, but not rats, treated with 2,4- or 2,6-DMA.

The above disparities may be the result of one or more causes. First, the methods employed in the present study, gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS), were different from that used by Lindstrom (paper chromatography). GC and GC/MS are highly sensitive and, in the case of the latter, highly specific. Paper chromatography can be difficult to interpret as compounds are identified on the basis of the distance of their migration along a paper sheet compared to the migration of standards. A certain degree of subjective interpretation is often required in comparing the migration of unknowns and standards. GC retention times, which are determined electronically, are more exact in this regard. Adequate resolution between multiple unknowns and/or substances naturally present in the sample can be difficult to achieve with paper chromatography. Capillary GC has a much greater separative capability than paper chromatography and, as a general rule, provides excellent resolution. The GC nitrogen-phosphorus specific detector is also considerably more sensitive in detecting low concentrations of unknowns than is paper chromatography. It has the additional advantage of responding only to those compounds which contain
nitrogen or phosphorus and fails to recognize those substances naturally present in the sample which do not contain these atoms. This aids in the resolution of compounds of interest from those which are not and increases the specificity of the method. GC/MS, in most instances, is able to positively identify compounds on the basis of retention times and molecular weight and/or pattern of breakdown. The identification of compounds with this method is more accurate than that obtainable with paper chromatography. Therefore, differences in methods used in the present study and those used by Lindstrom could have substantially affected the results. Secondly, the dose of 2,4- and 2,6-DMA administered in Lindstrom's studies (200 mg/kg) was different from that employed here (2,4-DMA, 117 mg/kg; 2,6-DMA, 262.5 mg/kg). Additionally, the duration of treatment in Lindstrom's studies was not adequately defined and may not correspond to that of the present study. Alterations in metabolism may occur with variations in dose and duration of treatment as the result of changes in pharmacokinetics, enzyme induction or toxicity. These factors may also have affected the results obtained. However, the results of the present study were in agreement with that of Lindstrom's concerning the major urinary metabolites of 2,4- and 2,6-DMA in the rat, N-acetyl-4-amino-3-methyl-benzoic acid and 4-hydroxy-2,6-DMA, respectively. A discussion of the results of this study with regard to the urinary metabolites of 2,4- and 2,6-DMA in the dog and the rat follows.

The major urinary excretory products of 2,4-DMA in the rat and dog detected in the present study were N-acetyl-4-amino-3-methyl-benzoic acid and 6-hydroxy-2,4-DMA, respectively. The unchanged parent compound
was also detected in significant amounts in the urine of both dogs and rats. Metabolites present in lesser quantities included N,2,4-trimethylaniline in the rat and 4-amino-3-methyl-benzoic acid, its glycine conjugate and N,2,4-trimethylaniline in the dog. The sulfate and glucuronide conjugates of the parent compound and the major urinary metabolites were detected in the urine of dogs and rats and of 4-amino-3-methyl-benzoic acid in the urine of dogs.

The above results indicate that the in vivo metabolism of 2,4-DMA was significantly different in the rat and dog. The lack of an acetylated metabolite in dog urine was not unexpected as dogs are relatively deficient in acetylation capability. Rats, on the other hand, are proficient in this respect and N-acetylation of various compounds is common in this species. Likewise, the 6-hydroxylation of 2,4-DMA by the dog and the lack of such hydroxylation in the 6-position by the rat also appeared to follow species specific metabolic pathways. Carnivores preferentially oxidize aniline in the ortho-, rather than para-, position; whereas rodents show a preference for hydroxylation in the para-position. This relationship appears to hold true for MOCA metabolism in the dog (o-hydroxylation) and MDA metabolism in the rat and rabbit (hydroxylation of the methylene bridge para to the nitrogen) as well. The preference of carnivores for ortho-hydroxylation may also account for the greater production of 6-hydroxy-2,4-DMA as compared to 4-amino-3-methyl-benzoic acid in the dog. Similarly, the preference in rodents for para-oxidation may have been reflected in the production of the N-acetylated-benzoic acid derivative. As the para position of
2,4-DMA is occupied by a methyl group, para-oxidation must produce the alcoholic or carboxylic acid derivative.

It is tempting to ascribe the hepatotoxic effect of 2,4-DMA to N-acetyl-4-amino-3-methyl-benzoic acid. This compound was the major urinary excretory product of 2,4-DMA in the rat and was not identified in the urine of treated dogs. The cytoplasmic change attributed to 2,4-DMA treatment was more pronounced in rats receiving 3MC in addition to 2,4-DMA. This treatment group tended to excrete greater quantities of the N-acetyl-benzoic acid derivative as compared to animals receiving 2,4-DMA alone. A trend toward less excretion of this metabolite and a decrease in the prominence of the cytoplasmic change was observed in animals receiving PB or SKF-525A in addition to 2,4-DMA, compared to the 3MC:2,4-DMA treatment group. Furthermore, an N-acetylated-benzoic acid derivative was not identified in the urine of rats treated with 2,6-DMA. However, N-acetyl-4-amino-3-methyl-benzoic acid does not appear to be an extremely reactive chemical species and was found to be quite stable on storage in this laboratory.

If the N-acetyl-benzoic acid derivative was involved in the toxicity of 2,4-DMA, at least two possible modes of action exist. First, N-acetyl-4-amino-3-methyl-benzoic acid may have been converted to the N-hydroxy species in the liver. Metabolic activation of 2-acetylaminofluorene, for example, proceeds through N-hydroxylation and subsequent action by sulfotransferase, N-O-acyltransferase and/or deacetylase.117 N-hydroxy-2,4-DMA has been detected after in vitro incubation of rat liver S9 fractions, proving that N-hydroxylation of at
least the parent compound is possible in this species. The possible existence of N-hydroxy-N-acetyl-3-methyl-benzoic acid should not be discounted merely because it has not been detected in the urine of treated rats. This compound, or metabolites resulting from its further biotransformation, may be highly reactive species with very brief half lives or high capability for binding to cellular macromolecules. Second, the synthesis of N-acetyl-4-amino-3-methyl-benzoic acid in vivo proceeds through several steps and requires the concerted action of several enzymes. An acetyl group is added at some point in its synthesis and the methyl group in the 4 position is oxidized to carboxylic acid (-COOH) through the successive actions of microsomal oxidase, alcohol dehydrogenase and aldehyde dehydrogenase. It is possible that a reactive chemical species is produced somewhere along this metabolic pathway. Possible reactive species include 4-amino-3-methyl-benzyl alcohol, 4-amino-3-methyl-benzaldehyde or their acetylated or sulfate ester conjugates. The sulfate ester of the carcinogen 5-hydroxymethylchrysene has been found to be mutagenic and bind to calf thymus DNA in vitro. Binding to DNA appeared to occur through the -CHO moiety following the loss of the sulfate anion. 2-Amino-benzalcohol or a further metabolite, possibly the sulfate ester, appears to involved in the covalent binding of 2-nitrotoluene to rat liver DNA. Allyl alcohol, a periportal hepatotoxicant, is converted via alcohol dehydrogenase to the aldehyde acrolein which is a toxic alkylating agent. The above examples lend support to the hypothesis that the alcoholic or aldehyde metabolites of 2,4-DMA or their N-acetylated derivatives may have been involved in the hepatotoxicity of
the parent compound.

Alternately, a highly reactive metabolite not related to N-acetyl-4-amino-3-methyl-benzoic acid and not detected in urine may be responsible for the toxicity of 2,4-DMA. 2,4-DMA has been shown to increase hepatic smooth endoplasmic reticulum, microsomal protein and cytochrome P-450 content in rats. This would indicate the potential for an alteration in the metabolism of 2,4-DMA, although no such change was observed in this study with respect to urinary metabolites. The possibility that 2,4-DMA metabolism may be altered with time to produce minute quantities of a highly reactive species is not negated because such a product was not detected in urine.

With respect to the metabolism of 2,6-DMA, the major excretory product detected in both rat and dog urine was 4-hydroxy-2,6-DMA. The unchanged parent compound was detected in significant amounts in the urine of both dogs and rats. Metabolites present in lesser quantities included N,2,6-trimethylaniline in the rat and 2-amino-3-methyl-benzoic acid, 2,6-dimethyl-nitrosobenzene, the glycine conjugate of 2-amino-3-methyl-benzoic acid, N,2,6-trimethylaniline and an unknown tentatively identified as 4-imino-3,5-dimethyl-quinone in the dog.

Differences in the metabolism of 2,6-DMA by the dog and rat were not as great as those seen with 2,4-DMA. The major urinary excretory product of 2,6-DMA was identical in the dog and rat, whereas the major urinary excretory products of 2,4-DMA in the dog and the rat were of totally different chemical structure. However, as with treatment with 2,4-DMA,
the dog produced a greater variety of 2,6-DMA metabolites than the rat.

With respect to the urinary metabolites identified in the dog, there are two logical possibilities for the cause of the hepatotoxicity, i.e. 4-hydroxy-2,6-DMA and the unknown tentatively identified as 4-imino-3,5-dimethyl-quinone. 4-Hydroxy-2,6-DMA deserves consideration as it was the major excretory product of 2,6-DMA isolated in dog urine. However, 4-hydroxy-2,6-DMA was also the major urinary metabolite of 2,6-DMA in the rat, a species where little or no hepatic change was observed in response to 2,6-DMA. One possible explanation for the difference in response, if 4-hydroxy-2,6-DMA was involved in the toxicity of 2,6-DMA, would be a species difference in susceptibility. Dogs were obviously more sensitive to the hepatotoxic effect of 2,6-DMA than the rat. The no effect dose of 2,6-DMA in the rat (262.5 mg/kg) was approximately 10 times greater than that which induced fatty degeneration in the dog (25 mg/kg) in this study. Therefore, it is possible that dogs are more sensitive to 4-hydroxy-2,6-DMA than the rat. In support of the hypothesis that rats may be relatively insensitive to any hepatotoxic effects of 4-hydroxy-2,6-DMA is the finding that dimethyl substitution of acetaminophen (N-acetyl-4-hydroxy-benzene) in the 2 and 6 positions substantially reduced the hepatotoxicity produced by acetaminophen or 3,5-dimethyl-acetaminophen in rats and mice.134 A second explanation for a possible difference in response to 4-hydroxy-2,6-DMA in the rat and the dog would be the metabolic route by which this metabolite was produced. 4-Hydroxy-2,6-DMA could arise via direct hydroxylation or by epoxidation followed by nonenzymatic rearrangement. Arene oxides are thought to be involved in the toxicity
of a variety of chemical compounds by acting as electrophilic intermediates which covalently bind to DNA, RNA and protein.\textsuperscript{135,136,137} Examples of compounds for which the synthesis of arene oxides is important in the production of toxicity, mutagenesis and/or carcinogenesis include benzo[a]pyrene, vinyl chloride, bromobenzene, benzene, 2,5,2',5'-tetrachloro-biphenyl, furosemide and aflatoxin B1.\textsuperscript{136,137} The dog may produce a significant amount of the 4-hydroxy metabolite via epoxidation of the parent compound, whereas the rat may synthesize 4-hydroxy-2,6-DMA via direct hydroxylation. Alternately, production of the 4-hydroxy metabolite may proceed via epoxidation in both species, but the rat may be more efficient at detoxifying this intermediate through epoxide hydrolase or glutathione conjugation than the dog. The balance between activation and detoxification is a critical determinant in the ultimate production of toxicity.\textsuperscript{114,115,116}

In support of the hypothesis that 4-hydroxy-2,6-DMA was responsible for the hepatotoxicity of 2,6-DMA was the finding that dogs initially converted a relatively greater amount of the available 2,6-DMA to the hydroxylated compound than the rat. The molar ratio of metabolite to parent on Day 1 was 7.39 $\pm$ 3.71 in the dog compared to 2.05 $\pm$ 0.36 in the rat. Following ten days of treatment these values were roughly equivalent in the two species (3.19 $\pm$ 2.53 vs. 2.58 $\pm$ 0.59 in the dog and rat, respectively). The trend in dogs toward a decrease in the production of 4-hydroxy-2,6-DMA may have been a manifestation of the resulting hepatotoxicity.
In further support of the above postulate was the appearance of mild lipid vacuolization in rats treated with PB or 3MC in conjunction with 2,6-DMA. A significant increase in the production of 4-hydroxy-2,6-DMA was observed on Day 10 in rats receiving 3MC as compared to those treated with 2,6-DMA alone. A trend toward greater production of the metabolite was also seen on Day 1 and Day 5 in rats treated with PB and 2,6-DMA.

Both 2,6- and 2,4-DMA give rise to hydroxylated compounds as the major urinary metabolites in the dog. The production of 4-hydroxy-2,6-DMA has been proposed in this discussion as a possible explanation for the induction of hepatotoxicity in the dog. By inference, the isomer of the above compound, 6-hydroxy-2,4-DMA, did not appear to be toxic as evidenced by the lack of hepatic change on histopathologic examination. On first consideration it would appear unlikely that the two isomers could produce such divergent hepatic responses. However, an obvious difference exists between the effects of the parent isomers, 2,6- and 2,4-DMA, in the dog. Furthermore, differences have also been observed in the toxicity of isomers of the hydroxylated metabolites of 2-acetyl-aminoflourene. Therefore it is entirely possible that 4-hydroxy-2,6-DMA, but not 6-hydroxy-2,4-DMA, is hepatotoxic in the dog.

Alternately, the unknown tentatively identified as 4-imino-3,5-dimethyl-quinone, which was found in the urine of dogs but not rats, may have been responsible for the hepatotoxicity of 2,6-DMA observed in the dog. Quinones have been implicated in the toxicity of a
variety of aromatic compounds. One of the most thoroughly studied compounds in this regard is acetaminophen. Acetaminophen is metabolized to N-acetyl-p-benzo-quinone imine (NAPQI) by the action of hepatic cytochrome P-450 or via prostaglandin synthetase in a hydroperoxidase-catalysed cooxidation reaction. The quinone imine is highly reactive both as an electrophile and as an oxidant, and because of these properties covalently binds to tissue proteins and may stimulate peroxidative events. The net result is the induction of hepatic necrosis. In addition, the dimethyl substituted derivative of NAPQI, N-acetyl-2,6-dimethyl-p-benzo-quinone imine, has been found to bind glutathione and to produce a free radical intermediate on reduction by NADPH-cytochrome P-450 reductase in vitro. These findings imply that this compound may also be toxic in vivo. It is possible that the quinone imine derivative of 4-hydroxy-2,6-DMA may also have toxic properties similar to those of NAPQI and N-acetyl-2,6-dimethyl-p-benzo-quinone imine.

Covalent Binding

The covalent binding of [14C]-2,4-DMA to hepatic DNA, RNA or protein was consistently higher than that observed for [14C]-2,6-DMA. These results support the contention that a reactive metabolite of 2,4-DMA is involved in its hepatotoxicity and are consistent with the differences observed in the urinary metabolites of 2,4- and 2,6-DMA in the rat.

The higher binding to protein by [14C]-2,4-DMA (21.93 ± 2.69 pM/mg) compared to RNA and DNA (9.60 ± 2.04 pM/mg and 2.11 ± 0.95 pM/mg,
respectively) was not unexpected. The cellular concentrations of protein are much greater than that of RNA and DNA so that a random encounter with a reactive compound is much more likely. Protein is also the major constituent of cytochrome P-450 and other drug metabolizing enzymes. Binding at the site of production of a reactive metabolite is likely and probable. [14C]-2,4-DMA covalent binding to protein was not, however, accompanied by a decrease in the concentration of hepatic protein which would indicate a destructive event. An increase in protein concentration was seen in response to 2,4-DMA treatment compared to control and 2,6-DMA animals. Therefore, an increase in the synthesis or a decrease in the catabolism or export of hepatic protein probably occurred. An increase in the synthesis of protein would be consistent with the ability of 2,4-DMA to cause enzyme induction.47,118,119 Binding of 2,4-DMA or a metabolite of 2,4-DMA may be an important stimulus leading to enzyme induction. The effect of 2,4-DMA on the catabolism or export of hepatic protein has not been investigated.

The degree of binding of [14C]-2,4-DMA to hepatic RNA was less than binding to protein and may be a reflection of the smaller quantities of RNA available within the cell. The higher RNA binding of 2,4-DMA, compared to 2,6-DMA, was consistent with the effect of 2,4-DMA on the rough endoplasmic reticulum observed with electron microscopy. Hepatic RNA concentrations were also significantly reduced by 2,4-DMA treatment compared to Control and 2,6-DMA animals. This was an unexpected finding as protein concentrations were increased in these animals and adequate levels of functional RNA are required for protein synthesis. It is possible that the segregation of RER observed with electron microscopy
may have affected the extraction process so that not all of the RNA within the sample was recovered. However, since increased binding to RNA was found along with electron microscopic changes and a decrease in RNA concentration, it appears likely that 2,4-DMA exerted a toxic effect on hepatic RNA. Several possibilities may be proposed to reconcile the decreased RNA concentrations with the higher protein levels: 1) the decrease in RNA content may be a late response, occurring after the synthesis of new protein; 2) 2,4-DMA may enhance RNA activity so that less total RNA is required; or 3) 2,4-DMA may decrease the catabolism or export of hepatic protein and thus increase protein concentration without stimulating new protein synthesis.

The degree of $[^{14}\text{C}]-2,4$-DMA binding to hepatic DNA was less than that to protein or RNA. Cellular concentrations of DNA are normally much lower than that of protein and RNA. Isolation of DNA within the nuclear membrane would require that a reactive compound traverse this membrane before interacting with DNA. Both of these factors probably contributed to the lower binding to DNA compared to RNA or protein. DNA covalent binding by $[^{14}\text{C}]-2,4$-DMA was higher than that of $[^{14}\text{C}]-2,6$-DMA as was the calculated covalent binding index (CBI). This finding is consistent with the mutagenic activity of 2,4-DMA on the Ames test.\textsuperscript{19,50,51} DNA binding was not, however, extremely high for either compound compared to potent hepatocarcinogens. In his paper evaluating DNA covalent binding and chemical carcinogenesis, Lutz stated that a CBI in the thousands represented strong hepatocarcinogens, a CBI in the hundreds represented moderate hepatocarcinogens and compounds with a CBI in the tens were weak hepatocarcinogens.\textsuperscript{23} The low CBI
of both 2,4- and 2,6-DMA would place these compounds in the latter category. This result is consistent with that found in carcinogenesis bioassays where neither 2,4- nor 2,6-DMA produced hepatic tumors.\(^{43,52}\) 2,6-DMA was, however, effective in inducing tumors of the nasal turbinates.

**Summary and Conclusions**

1.) 2,4-DMA, administered by gavage at 117 mg/kg/day for 10 days in corn oil, induced histopathologic and electron microscopic lesions in the livers of treated rats. Lesions observed with the light microscope were subtle but distinct and consisted of a pale voluminous cytoplasm and segregation or clumping of subcellular organelles. The combined effects of SER hyperplasia, polyribosome aggregation, and RER islands observed with the electron microscope were felt to be responsible for the subtle 2,4-DMA lesion seen on light microscopy.

2.) 2,6-DMA, administered by gavage at 262.5 mg/kg/day for 10 days in corn oil, was essentially without effect on the livers of treated rats. Light and electron microscopic sections from these animals were considered to be within the range of normal.

3.) 2,4-DMA, administered orally to dogs at 25 mg/kg/day for 10 days, produced no significant hepatic lesions in treated animals. Evaluation of hepatic sections with the light and electron microscope showed them to be within the range of normal.
4.) 2,6-DMA, administered orally to dogs at 25 mg/kg/day for 10 days, induced a moderate to severe hepatic centrilobular fatty degeneration in treated animals. The accumulation of lipid in affected cells was the most prominent change observed on both light and electron microscopic evaluation.

5.) 2,4-DMA treatment significantly increased the liver weight and the liver:body ratio, but had no effect on the total body weight of rats when compared to corn oil gavaged controls.

6.) 2,6-DMA treatment had no effect on body weight, liver weight or the liver:body weight ratio in rats compared to corn oil gavaged controls.

7.) 2,4-DMA was excreted in the urine of treated rats primarily as N-acetyl-4-amino-3-methyl-benzoic acid, the parent compound and as the sulfate or glucuronide conjugates of these compounds. N,2,4-trimethylaniline was detected in minor amounts.

8.) 2,6-DMA was excreted in the urine of treated rats primarily as 4-hydroxy-2,6-DMA, the parent compound and the sulfate and glucuronide conjugates of both of these compounds. N,2,6-trimethylaniline was detected in minor amounts.

9.) 2,4-DMA was excreted in the urine of treated dogs primarily as 6-hydroxy-2,4-DMA, the parent compound, 4-amino-3-methyl-benzoic acid and the sulfate and glucuronide conjugates of these compounds. N,2,4-trimethylaniline and the glycine conjugate of
4-amino-3-methyl-benzoic acid were detected in minor amounts.

10.) 2,6-DMA was excreted in the urine of treated dogs primarily as 4-hydroxy-2,6-DMA, the parent compound, 2-amino-3-methyl-benzoic acid and as the sulfate and glucuronide conjugates of these compounds. An unknown, molecular weight 135 and tentatively identified as 4-imino-3,5-dimethyl-quinone, was also detected. N,2,6-trimethylaniline, 2,6-dimethylnitrosobenzene, and the glycine conjugate of 2-amino-3-methyl-benzoic acid were detected as minor metabolites.

11.) Concurrent administration of PB and 2,4-DMA for 5 days to rats resulted in the mortality of 50% of the treatment group by Day 5. No histopathologic or electron microscopic lesions distinct to this treatment group were found. No major change compared to rats receiving 2,4-DMA only was observed for urinary metabolites.

12.) Concurrent administration of 3MC and 2,4-DMA to rats enhanced the 2,4-DMA lesion observed on light and electron microscopy. Liver weight and liver:body weight ratios were increased over those of the 3MC:Control group. A trend toward greater production of the N-acetyl-benzoic acid metabolite and less excretion as parent compound was observed.

13.) Concurrent administration of PB and 2,6-DMA for 5 or 10 days to rats significantly reduced body weights from that of control animals. No histopathologic or electron microscopic lesions distinct to this
treatment group were found. A trend toward greater production of the 4-hydroxy metabolite and less excretion as parent compound was observed.

14.) Concurrent administration of 3MC and 2,6-DMA for 10 days induced no significant change in the histopathologic or electron microscopic appearance from that of 3MC:Contol animals or those treated solely with 2,6-DMA. A slight increase in lipid vacuolization was observed. 3MC treatment tended to increase the excretion of 4-hydroxy-2,6-DMA at the expense of excretion as the parent compound.

15.) Administration of SKF-525A, at 50 mg/kg/day via IP injection for 10 days, induced a hepatic midzonal fatty degeneration in the livers of treated rats. Concurrent administration of 2,4-DMA or 2,6-DMA minimized or lessened, respectively, the midzonal lipid accumulation due to SKF-525A treatment. SKF-525A tended to increase the urinary excretion of 2,4- or 2,6-DMA as the parent compounds and decrease the excretion of their major urinary metabolites.

16.) Concurrent administration of SKF-525A and 2,4-DMA to rats diminished the characteristic 2,4-DMA cytoplasmic lesion, but had no effect on the ability of 2,4-DMA to alter liver weight or the liver:body weight ratio.

17.) The covalent binding of $^{14}$C]-2,4-DMA, expressed as CBI or picomoles bound/mg, to rat hepatic DNA, RNA or protein was significantly higher than that of $^{14}$C]-2,6-DMA following a 9 day pretreatment with unlabelled compounds. Hepatic protein and RNA concentrations were
significantly increased and decreased, respectively, by 2,4-DMA treatment. DNA concentrations were unaffected. 2,6-DMA treatment did not alter the hepatic DNA, RNA or protein concentrations.
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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Marcia L. Hardy

Major Field: Veterinary Medical Sciences (Toxicology Option)

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Approved:

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Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

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