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A STUDY OF THE EFFECT OF HYDROCARBON STRUCTURE ON THE
INDUCTION OF MALE RAT NEPHROPATHY AND METABOLIC
STRUCTURE

Submitted By:

M. Paul Serve'

Department of Chemistry

Wright State University

Dayton OH 45435

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ABSTRACT

Certain hydrocarbons have been shown to cause nephrotoxicity in male rats. Since many of the hydrocarbons have a branched alkyl side chain, several isomers of octane including 2,5-dimethylhexane, 2-methylheptane, 3-methylheptane and 4-methylheptane were evaluated on their ability to induce the nephrotoxicity. Since the above hydrocarbons are components of fuels and solvents, an investigation into their pathways of metabolism was undertaken in order to see if a pattern of biotransformation could be deduced and determine if potentially harmful metabolites were produced. Male 344 Fischer rats were dosed intragastrically with the above hydrocarbons over a 14 day period. When compared with control rats, the following order of nephrotoxicity was determined: n-octane < 4-methylheptane $\pm$ 2-methylheptane < 3-methylheptane < 2,5-dimethylhexane < 2,2,4-trimethylpentane < 2,3,4-trimethylpentane. The metabolites identified for the following compounds were:

2,5-Dimethylhexane: 2,5-dimethyl-1-hexanoic acid, 2,5-dimethyl-1,2-hexanediol, 2,5-dimethyl-1,5-hexanediol, 2,5-dimethyl-2,5-hexanediol and 2,5-dimethyl-5-hydroxy-1-hexanoic acid.

2-Methylheptane: 2-methyl-1,2-heptanediol, 2-methyl-1,5-heptanediol, 2-methyl-2,5-heptanediol, 2-methylheptanoic acid and 2-methyl-5-hydroxy-1-heptanoic acid.

3-Methylheptane: 3,5-diethyl-2,3-dihydrofuran, 3-ethyl-6-methyl-2,3-dihydropyran, 3-methyl-3-heptanol, 3-methyl-2-heptanol, 5-methyl-2-heptanol, 2-n-butyl-1,3-butanediol, 2-ethyl-1,3-hexanediol, β -methyl- δ -enantholactone, 3-methyl-3,4-heptanediol, 3-methyl-2,3-heptanediol, 3-methyl-3,5-heptanediol, 2-ethyl-1,4-hexane-diol, δ -methyl- δ -enantholactone, 5-methyl-2,5-heptanediol, 2-ethyl-1,5-hexanediol and 2-ethylhexanoic acid (2-EHA).

4-Methylheptane: 6-hydroxy-4-methyl-2-heptanone, 4-methyl-3,5-heptanediol, di-n-propyl acetic acid, 4-methyl-2,5-heptanediol, 2-methyl-3-hydroxy-1-pentanoic acid, 4-methyl-1-heptanoic acid, 4-methyl-1,4-heptanediol, 4-methyl-2,6-heptanediol and 4-methyl-1,6-heptanediol.

Submitted By: M. Paul Serve'
Department of Chemistry
Wright State University
Dayton OH 45435

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ABSTRACT

Certain hydrocarbons have been shown to cause nephrotoxicity in male rats. Since many of the hydrocarbons have a branched alkyl side chain, several isomers of octane including 2,5-dimethylhexane, 2-methylheptane, 3-methylheptane and 4-methylheptane were evaluated on their ability to induce the nephrotoxicity. Since the above hydrocarbons are components of fuels and solvents, an investigation into their pathways of metabolism was undertaken in order to see if a pattern of biotransformation could be deduced and determine if potentially harmful metabolites were produced. Male 344 Fischer rats were dosed intragastrically with the above hydrocarbons over a 14 day period. When compared with control rats, the following order of nephrotoxicity was determined: n-octane < 4-methylheptane $\leq$ 2-methylheptane < 3-methylheptane < 2,5-dimethylhexane < 2,2,4-trimethylpentane < 2,3,4-trimethylpentane. The metabolites identified for the following compounds were:

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2-Methylheptane: 2-methyl-1,2-heptanediol, 2-methyl-1,5-heptanediol, 2-methyl-2,5-heptanediol, 2-methylheptanoic acid and 2-methyl-5-hydroxy-1-heptanoic acid.

3-Methylheptane: 3,5-diethyl-2,3-dihydrofuran, 3-ethyl-6-methyl-2,3-dihydropyran, 3-methyl-3-heptanol, 3-methyl-2-heptanol, 5-methyl-2-heptanol, 2-n-butyl-1,3-butanediol, 2-ethyl-1,3-hexanediol, β -methyl- δ -enantholactone, 3-methyl-3,4-heptanediol, 3-methyl-2,3-heptanediol, 3-methyl-3,5-heptanediol, 2-ethyl-1,4-hexane-diol, δ -methyl- δ -enantholactone, 5-methyl-2,5-heptanediol, 2-ethyl-1,5-hexanediol and 2-ethylhexanoic acid (2-EHA).

4-Methylheptane: 6-hydroxy-4-methyl-2-heptanone, 4-methyl-3,5-heptanediol, di-n-propyl acetic acid, 4-methyl-2,5-heptanediol, 2-methyl-3-hydroxy-1-pentanoic acid, 4-methyl-1-heptanoic acid, 4-methyl-1,4-heptanediol, 4-methyl-2,6-heptanediol and 4-methyl-1,6-heptanediol.

I. GENERAL INTRODUCTION

Hydrocarbon-induced nephropathy has become of great concern since inhalation of unleaded gasoline vapors following a 2-year exposure was shown to cause renal lesions, including carcinomas, in male rats [1]. Kidney damage, consisting initially of an accumulation of hyaline (a translucent albuminoid substance) droplets in proximal tubular cells, was seen in male rats, but not in female rats or in either sex of other rodents tested. In addition to gasoline vapors, other hydrocarbons found to produce similar renal lesions include Stoddard solvent (a mixture of aliphatic hydrocarbons with some naphthene and benzene derivatives) [2], 2,2,4-trimethylpentane (isooctane, 2,2,4-TMP) [3], 2,3,4-trimethylpentane (2,3,4-TMP) [4], the petroleum-based aviation fuels JP-4 and RJ-5 [3], the synthetic Cruise Missile fuel JP-10 [3], decalin [3] (decahydronaphthalene) and tetralin (tetrahydronaphthalene) [5]. Other hydrocarbon molecules which possess both a ring structure and a branched chain, such as isopropylcyclohexane (i-PCH) [6] and tertiary-butylcyclohexane (t-BCH) [7] have also been shown to induce this kidney damage. Non-saturated alkanes which have been reported to produce this hyaline droplet nephropathy include d-limonene [8], isophorone [9] and 1,4-dichlorobenzene [10]. Chronic exposure of male rats to the chemicals above results in the induction of neoplasms in up to 25% of the animals [11]. When tested in other species, including mice, hamsters, guinea pigs, dogs and non-human primates, these chemicals did not produce any pathological effects on the kidneys. The relevance of this pathology in male rats to the possible production of similar lesions in humans is therefore important. While epidemiologic studies do not definitely indicate an increased incidence of nephropathy in humans exposed to gasoline vapors, there are surveys suggestive of a positive association [12].

There are differences in the metabolism of xenobiotics between species and between the sexes. As a result of the chronic gasoline inhalation study that demonstrated nephropathy and carcinogenicity in male rats, a Workshop on the Kidney Effects of Hydrocarbons was held in Boston in July 1983. A major recommendation made at this workshop was to investigate the metabolism of known nephrotoxic hydrocarbons [13]. Because of this recommendation by toxicologists and because of the fact that millions of people are exposed daily to hydrocarbons in fuels, solvents and varnishes, large research investments to investigate this potential problem have been made by the American Petroleum Institute, General Motors and the Chemical Industry Institute of Toxicology [14].

Biochemical and pathophysiology studies have revealed that the hyaline droplets are an accumulation of $\alpha_2\text{u}$ -globulin in protein droplets (lysosomes) [15, 16]. Soon degeneration and necrosis of cells lining the P(2) segment of the proximal tubule occur. This is followed by the formation of granular casts at the junction of the thin loop of henle and the P(3) segment of the proximal tubule. After a period of time regeneration of proximal tubular epithelium occurs.

$\alpha_2\text{u}$ - GLOBULIN

$\alpha_2\text{u}$ -globulin is a low molecular weight protein that is synthesized by the parenchymal cells of the liver of the adult male rat and secreted into the blood at a rate of 90-180 ug/g of liver/hr [17, 18, 19]. Its synthesis is under multihormonal control, with androgens being required, but growth hormone, glucocorticoids and thyroxine also influencing the process [20, 21]. Since $\alpha_2\text{u}$ has a molecular weight of 18,700 daltons, it is freely filtered through the glomerulus. The kidneys of an adult male rat filter 50-60 mg/day of $\alpha_2\text{u}$ with 40% remaining in the filtrate and being excreted in the urine and 60% being reabsorbed by the kidney [22]. Female rats excrete less than 1% of the $\alpha_2\text{u}$ excreted by male rats [23]. In contrast to the male rat which synthesizes nearly all of its $\alpha_2\text{u}$ in the liver, the female rat $\alpha_2\text{u}$ is thought to be of salivary gland origin.

The amino acid sequence of $\alpha_2\text{u}$ -globulin indicates that the hepatic form is composed of 162 amino acids [24]. The $\alpha_2\text{u}$ found in the urine is similar to the 162 amino acid hepatic form, while the $\alpha_2\text{u}$ found in the kidney is 11 amino acids shorter. Nine and two amino acids are cleaved

from the N-terminal and C-terminal parts of the protein respectively [25]. Injecting α_2u labeled with [3H] leucine, into control male rats, a half-life of 5 hours was found [25].

The reabsorption and processing of α_2u in the male rat kidney are similar to that of other low molecular weight proteins [26, 27]. The protein is taken into the epithelial cells of the P2 segment by endocytosis. The endocytotic vesicles fuse with phagolysosomes, where the proteins are hydrolyzed by the lysosomal enzymes. Amino acids liberated by this process are returned to the general circulation, while undigested material is either excreted into the nephron lumen or accumulates in the cell. Immunohisto chemistry on perfusion-fixed male and female control rat kidneys utilizing a monoclonal antibody to α_2u clearly demonstrated that α_2u was localized in small round organelles thought to be lysosomes in males. α_2u was not detectable in female rats using histo chemical staining [28]. When α_2u from male rats was partially purified and injected into female rats, protein droplets were observed.

α_2u - GLOBULIN SUPERFAMILY OF PROTEINS

Recently, a number of low-molecular weight proteins have been classified into one family of proteins based on computer generated sequence alignment of their primary protein structures [29, 30]. Included among the 10 distinct members of this family of proteins are: human and rat retinol binding protein (RBP), bovine B-lactoglobulin (LG), rat α_2u , mouse major urinary protein (MUP), human protein (HC), human and rat α_1 -acid glycoprotein (AGP), and human apolipoprotein D. Comparison of RBP and LG 3-dimensional structures indicates that they are quite similar in that they both contain an eight stranded beta-barrel core that encloses the protein [31]. All of the proteins in this superfamily are either known or proposed to be transport proteins because of their ability to bind hydrophobic compounds to varying degrees. The interaction of proteins in this superfamily with chemicals that cause α_2u nephropathy in male rats, the amount of protein synthesized and filtered by the glomerulus and the efficiency of protein hydrolysis may be critical factors involved in species extrapolation of risk from chemicals causing α_2u -nephropathy.

METABOLISM OF CHEMICALS WHICH PRODUCE α_2 -GLOBULIN NEPHROPATHY

It is well accepted that a hydrocarbon is a neutral molecule and not capable of forming strong bonds with a protein such as alpha-2u globulin. Biotransformation of the hydrocarbon by various oxidation enzyme systems (e.g., cytochrome P-450 enzymes) to a molecule with polar substituents (e.g., alcohol, carboxylic acid or ketone groups) would permit the hydrocarbon metabolite to form strong hydrogen bonds with an indigenous protein. The report by Olson et.al. on work, sponsored by the Air Force, which indentified the urinary metabolites of 2,2,4-TMP [32] spurred interest in determining if the metabolic structure of a molecule could be related to the molecule's ability to induce renal damage [33, 34].

Previous studies on the metabolism of various types of saturated hydrocarbons indicate that the presence or absence of a ring, a branched methyl group, or a long, straight-chain hydrocarbon can profoundly alter structures of the urinary metabolites found. An examination of saturated cyclic hydrocarbon (cis- and trans-decalin, and tetralin) metabolism revealed that the urinary metabolites were alcohols, hydroxy ketones and diols [5, 35]. The biotransformation of acyclic hydrocarbons containing penultimate methyl groups (2,2,4-TMP and 2,3,4-TMP) resulted in the urinary excretion of alcohols, carboxylic acids, and hydroxy acids [4, 32]. The metabolism of straight-chain, saturated hydrocarbons, which are very similar in physical and chemical properties to chemicals which cause nephropathy, seem to yield different sets of urinary metabolites. Metabolism of n-hexane yielded alcohols and diketones which contain six carbon atoms. On the other hand, n-octane was biotransformed to alcohol, carboxylic acid and ketoacid derivatives containing six to eight carbons [36]. This cleavage of carbon-carbon bonds appears to be a rarity in saturated hydrocarbon metabolism. The ability for n-octane to be shortened in length during metabolism may be important in explaining why this straight-chain hydrocarbon does not produce hyaline droplet nephropathy. The resultant six-carbon metabolite of n-octane is a very polar, highly excretable keto acid. A shortened stay in the body may preclude either the hydrocarbon or a metabolite of the hydrocarbon from inducing the nephropathy. The fact that n-hexane does not induce nephrotoxicity may be as much attributable to its physical properties as its chemical structure.

The low boiling point of n-hexane (84°C) may permit the animal to volatilize much of the chemical and reduce the amount which must be metabolized. Hydrocarbons which have been demonstrated to cause male rat nephropathy contain 8 to 12 carbon atoms. These hydrocarbons have boiling points in the range of 120 - 200°C.

Using [¹⁴C] 2,2,4-TMP, it was shown that selective retention of radioactivity occurred in the male rat kidney, but not the female rat kidney 72 hr. after dosing [37]. More detailed investigations revealed that 2,2,4-TMP reached its peak concentration in the kidney within 12 hr. of administering a single dose of [¹⁴C]2,2,4-TMP to male or female rats [33]. The peak concentration in kidney of male rats was 2X that of females, while no differences were noted for liver or plasma. The 2,2,4-TMP dose-response relationship was nonlinear with a greater percentage of the dose being retained at low doses. This suggested that the mechanism responsible for retention of 2,2,4-TMP reactivity was saturable. α_2u also increased in male rat kidney in a dose-time-related fashion, while it remained below the limit of detection in female rat kidney [33, 38]. The major metabolite of 2,2,4-trimethyl-2-pentanol (TMPOH), while no metabolites were detected in female rats. In contrast, greater concentrations of TMPOH conjugated to glucuronic acid were found in female rat urine.

No covalent binding of 2,2,4-TMP or its metabolites to renal macromolecules was demonstrated in male rats. However, reversible binding of radiolabel [3H]-2,2,4-TMP to a protein fraction containing α_2u was found in male rat kidney cytosol [34]. The radiolabel remained bound to the protein during dialysis against phosphate buffer, Sephadex G-75 chromatography and lyophilization, but was lost when the dialysis buffer contained 0.1% sodium decyl sulfate (SDS). SDS is a detergent which affects the secondary and tertiary structure of proteins allowing the release of chemicals bound to proteins by other than covalent bonds (e.g. H-bonds). Gas chromatography/mass spectrometry (GC/MS) of an acidified ethyl acetate extract of pooled Sephadex G-75 fractions containing α_2u demonstrated that TMPOH was the 2,2,4-TMP metabolite that was bound to α_2u . Similar analyses of kidneys from male rats exposed to 50ppm 2,2,4-TMP or 300ppm unleaded gasoline revealed that TMPOH was the metabolite bound to α_2u [39]. Time course studies showed that the

TMPOH- $\alpha_2\text{u}$ complex was slowly cleared from male rat kidney, remaining at similar concentrations from 8 to 72 hr. after exposure.

The reversible bindings of 1,4-dichlorobenzene (1,4-DCB) and isophorone (IPH) to $\alpha_2\text{u}$ have also been investigated. Gel filtration of cytosol prepared from kidneys of rats treated with [^{14}C -1,4-DCB [40] or [^{14}C] IPH [41] showed that the radiolabel coeluted with $\alpha_2\text{u}$. Equilibrium dialysis in the presence or absence of SDS demonstrated that the radiolabel derived from these compounds was reversibly bound to $\alpha_2\text{u}$. GC/MS analysis identified the chemicals bound to $\alpha_2\text{u}$ from rats treated with 1,4-DCB as 1,4-DCB and 2,5-Dichlorophenol, while IPH was the only compound bound to $\alpha_2\text{u}$ in IPH-treated rats.

In the case of 2,3,4-TMP, although several metabolites found from kidney homogenates in either male or female Fischer 344 rats [4].

The studies of cyclic molecules revealed some interesting findings in the cases of kidney homogenate metabolites and renal damage. Decalin-dosed rats which suffered kidney damage also contained the 2-decalone metabolite. The stereochemistry of the decalin did not seem to have any effect on the extent of renal damage [3]. Indan, which also induced a milder type of nephrotoxicity in male rats yield the molecules 1-indanol and 1-indanone from the kidney homogenate extracts [42]. Tetralin, which produced more renal damage than indan, did not yield any tetralin metabolites from kidney homogenate extracts [5].

EFFECT OF CHEMICAL BINDING OF METABOLITES ON PROTEIN CATABOLISM

It is well recognized that proteins differ in their susceptibility to hydrolysis by proteases [43]. It has recently become clear that protein half-lives, indices of protein catabolism by proteases, are determined by specific molecular determinants in the protein [44]. The role of the primary amino acid sequence seems to be a major factor, as shown by the observation of common peptide regions rich in proline, glutamate, serine and threonine (PEST regions) among rapidly degraded proteins.

Proteinase K hydrolysis of [^{14}C]($\alpha_2\text{u}$) purified from kidney cytosol prepared from male rats treated with 2,2,4-TMP (bound $\alpha_2\text{u}$) was markedly different from that observed for [^{14}C]($\alpha_2\text{u}$) purified from control male rat kidney cortisol (free $\alpha_2\text{u}$). Even though only 31+6% of the

bound α_{2u} represented TMPOH- α_{2u} complex, this preparation was hydrolyzed at a much slower rate than the free α_{2u} , such that after 48 hr. of incubation, there was a 25-30% reduction in hydrolysis of the bound α_{2u} . These data suggest that the actual TMPOH- α_{2u} complex is highly resistant to hydrolysis, even by a strong protease like proteinase K. Thus, the selective binding of TMPOH to α_{2u} seems to transform a poorly hydrolyzable protein to one that is virtually undigestible. A change in the conformational structure of α_{2u} when it binds to a hydrocarbon metabolite has been offered as an explanation as to why the α_{2u} suddenly becomes undigestible [45]. In the case of d-limonene, the binding of d-limonene-1,2-oxide caused a 5-fold reduction of α_{2u} degradation [46].

PATHOLOGICAL CONSEQUENCES OF α_{2u} -GLOBULIN ACCUMULATION

While the P(2) segment of renal epithelial cells of untreated male rats contain small round lysosomes that stain positively for α_{2u} , exposure to chemicals that cause α_{2u} nephropathy results in a dose-related accumulation of α_{2u} in large polyangular lysosomes. These abnormal organelles also stain positively for α_{2u} and are electron dense and frequently crystalloid when examined by electron microscopy [28, 47, 48]. Individual cells undergo cytolysis, detach from the basement membrane and slough into the lumen of the nephron. Some of these cells pass through to the urine, while in the case of severe α_{2u} nephropathy, others accumulate at the junction of the loop of Henle and the P(3) segment of the proximal tubule, forming granular casts. The loss of P(2) segment epithelial cells leads to a restorative hyperplasia of these cells. If this rapid regeneration of cells involves mutations, it is plausible that malignancies of the kidney will ensue. The extent of cell proliferation is dependent upon the dose, number of exposures, the age of the rat and the period of time elapsed from the last exposure [49, 50]. Cell proliferation was determined at 3, 6 or 12 months of exposure to unleaded gasoline in male and female rats. No increase in cell proliferation was detected in female rats regardless of site within the nephron, age of the animal, dose or number of exposures [50]. The amount of cell proliferation was greatest in male rats exposed for 3 months and least for those exposed for 12 months. This parallels the age-related synthesis of α_{2u} in male rats.

α_2 u - GLOBULIN SUMMARY

From the data gathered on α_2 u-globulin nephropathy, four steps seem to be apparent: (1) a reversible binding of a chemical (metabolite) to α_2 u alters the renal lysosomal. Catabolism of a poorly digested low-molecular weight protein to a further reduced state; (2) this leads to accumulation of the chemical- α_2 u complex in P(2) renal epithelial cells, causing lysosomal protein overload and individual cell necrosis; (3) this is followed by cell regeneration that continues in an exposure related extent for as long as the rat produces α_2 u; and (4) the increased amount of cell proliferation acts as a tumor promoter by clonally expanding spontaneously initiated cells in the kidney.

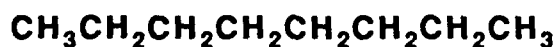
HYDROCARBON STRUCTURE AND ABILITY TO INDUCE MALE RAT NEPHROPATHY

As mentioned previously, not all hydrocarbons are capable of inducing the nephropathy. Also, it is highly unlikely that the hydrocarbon itself survives for a sufficient time to reach the kidney. A more plausible explanation is that the hydrocarbon is metabolized to derivatives which possess polar functionalities, which allow a binding to the α_2 u-globulin with the resulting alteration in the configuration of the renal protein. Obviously the structure of the hydrocarbon dictates the mode(s) of oxidative metabolism which the hydrocarbon can undergo.

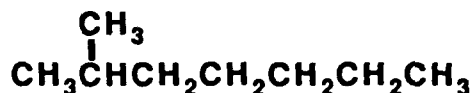
The different types of urinary metabolites found for various hydrocarbons suggest that either more than one pathway of oxidation exists, or that the structure of the hydrocarbon strongly controls the type of metabolic oxidation permitted. No studies of the structure - hydrocarbon nephropathy induction relationship have as yet been undertaken. An examination of several isomers of one hydrocarbon family should allow the determination of the influence of molecular structure on metabolism and the ability to cause renal damage.

n-Octane, 2-methylheptane (2-MH), 3-methylheptane (3-MH), 4-methylheptane (4-MH), 2,5-dimethylhexane (2,5-DMH), 2,2,3-trimethylpentane (2,2,3-TMP), 2,2,4-trimethylpentane (2,2,4-TMP), and 2,3,4-trimethylpentane (2,3,4-TMP) are all eight carbon isomers (Fig. 1). n-Octane is a simple, straight chain, saturate hydrocarbon, while the

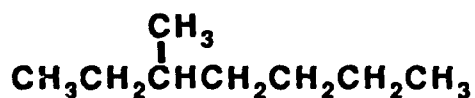
previously reported nephrotoxic hydrocarbons, 2,2,4-TMP and 2,3,4-TMP have alkyl groups, specifically methyl groups, at penultimate positions. The molecules 2-MH, 2,3,3-TMP, and 2,5-DMH have either one or both penultimate positions substituted with methyl groups. 2-MH, 3-MH, and 4-MH octane isomers differ only in the position of the methyl substituent. An investigation of the relative nephrotoxicity and the identification of urinary and kidney metabolites of 2-MH, 3-MH, 4-MH, and 2,5-DMH should help establish a structure-activity relationship and provide insight regarding the mechanism of hydrocarbon-induced nephropathy. If the hypothesis that the metabolic enzymes require at least one unsubstituted penultimate carbon in order to produce non-nephrotoxic metabolites is correct, than 2,5-DMH should be much more nephrotoxic than 2-MH, 3-MH and 4-MH. If the metabolic enzymes in the male rat require a chain of three unsubstituted carbons to approach the molecule for transformation (i.e. to prevent steric hindrance), 2-MH, 3-MH, and 4-MH should be much less nephrotoxic than 2,5-DMH. Elucidation of the comparable renal toxicity elicited by 2-MH, 3-MH, and 4-MH will yield specific data as to how a single methyl group substitution position can affect the extent of nephropathy.



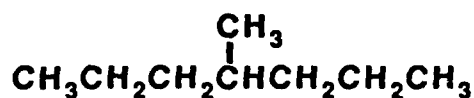
n-OCTANE



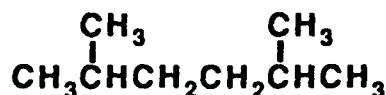
2-METHYLHEPTANE



3-METHYLHEPTANE



4-METHYLHEPTANE



2,5-DIMETHYLHEXANE

Investigation of the structures of the urinary metabolites of 2-MH, 3-MH, 4-MH and 2,5-DMH will provide insight on how the structure of a hydrocarbon impacts the ability of biotransformation enzymes to attack specific positions of the molecule. Because of the limited data available concerning the identification of urinary metabolite structure, developing a relationship between hydrocarbon and metabolite structures has not been previously attempted.

II. STATEMENT OF THE PROBLEM

To examine the effects of alkyl branching on saturated acyclic hydrocarbons* with respect to:

- A. The ability of the hydrocarbon to induce in male rats a nephropathy characterized by hyaline droplet formation, cast production and cell necrosis.
- B. The differences in structure of urinary metabolites produced by male and female rats exposed to the various hydrocarbons.
- C. The presence of any hydrocarbon metabolites residing in the kidneys of rats exposed to the hydrocarbons which may reveal information regarding the metabolic route or mechanism(s) of the induced nephropathy.
- D. The correlation between hydrocarbon structure, urinary metabolite structure and degree of nephrotoxic damage to gain an understanding of the progression of the nephropathy.

*For the purpose of brevity, hereafter, the term "branched-chain hydrocarbon" will refer to the following molecules; 2-methylheptane, 3-methylheptane, 4-methylheptane and 2,5-dimethylhexane.

Since the chemicals to be studied have separate and distinct physical, chemical and biological properties, each of the chemicals will be individually reported on the basis of background, experimental, results and conclusions. The following discussions of each of the chemicals evaluated for nephrotoxicity is a copy of the paper prepared for publication with respect to each of the hydrocarbons. The references in each of the individual papers as well as the figure numbers refer to the data in each of the specific papers. The references for the general introduction and conclusion sections are listed at the end of the report.

THE METABOLISM OF 2,5-DIMETHYLHEXANE IN MALE FISCHER 344 RATS

M. Paul Serve^a, Daniel D. Bombick^a, J Roberts^a, Gayle A. McDonald^a, David R. Mattie^b and Kyung O. Yub

^a Department of Chemistry, Wright State University, Dayton, OH 45435
and ^b Harry G. Armstrong Aerospace Medical Research Laboratory,
Wright-Patterson AFB, OH 45433.

ABSTRACT

The urinary metabolites of 2,5-dimethylhexane (2,5-DMH) in male Fischer 344 rats, administered the hydrocarbon by gavage, included 2,5-dimethyl-1-hexanoic acid, 2,5-dimethyl-1,2-hexanediol, 2,5-dimethyl-1,5-hexanediol, 2,5-dimethyl-2,5-hexanediol and 2,5-dimethyl-5-hydroxy-1-hexanoic acid. Metabolism favored the formation of the diols. The metabolites were identified using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Histopathologic examination revealed moderate hyaline droplet formation in the renal proximal tubule.

INTRODUCTION

2,5-Dimethylhexane (2,5-DMH), an isomer of octane, is a hydrocarbon found in fuel oils [1] and gasoline combustion exhaust [2]. A component of smog [3], 2,5-DMH has also been reported as being formed in structural fires [4], as a by-product from the extrusion of polyvinyl chloride [5] and as a constituent of tobacco fumes [6]. When injected into animals, isomers of octane have been shown to produce hemorrhage, edema and polymorphonuclear leukocytic reactions such as angitis, abscess formation thrombosis and fibrosis [7]. Certain branched chain isomers of octane such as 2,2,4-trimethylpentane (2,2,4-TMP) and 2,3,4-trimethylpentane (2,3,4-TMP) have previously been shown to be capable of inducing a proximal tubular nephropathy in male rats [8,9]. This nephropathy is characterized by the inability of the rat to breakdown the protein α_2 -globulin through

the normal lysosomal enzymes processes. 2,5-DMH, like other hydrocarbons, is a totally non-polar molecule. It is commonly accepted that for hydrocarbons to interfere in ongoing biochemical reactions, the molecule must be metabolized to various polar derivatives so that it can form bonds with cellular molecules.

Due to its ubiquitous nature in the environment and since many people will be exposed in various concentrations to 2,5-DMH, it was decided to examine the metabolic fate of 2,5-DMH in male rats. Male rats are frequently used to study the metabolism of xenobiotic materials because of the similarity of biotransformation reactions between rat and man. Secondly, a comparison of the ability of 2,5-DMH to 2,2,4- and 2,3,4-TMP to induce the male rat nephropathy would yield interesting information regarding the extent and positioning of structural branching necessary to induce the nephrotoxicity.

MATERIALS

2,5-Dimethylhexane, 2,5-dimethyl-1-hexene, 2,5-dimethyl-2,4-hexadiene, 2,5-dimethyl-2,5-hexanediol, 2-methylcyclopentanone and 2,2,4-trimethylcyclopentanone were purchased from Wiley Organics, Coshcoton, OH. 2,5-Dimethyl-1-hexanol was prepared by the hydroboration of 2,5-dimethyl-1-hexene (10). The oxidation of 2,5-dimethyl-1-hexanol to 2,5-dimethyl-1-hexanoic acid was accomplished using chromic acid (11). 2,5-Dimethyl-1,2-hexanediol was synthesized by treatment of 2,5-dimethyl-1-hexene with osmium tetroxide (12). The Baeyer-Villager oxidations of 2-methylcyclopentanone and 2,2,4-trimethylcyclopentanone yielded δ -methyl- δ -valerolactone and α,δ,δ -trimethyl- δ -valerolactone respectively (13). The reaction of δ -methyl- δ -valerolactone with methyl magnesium iodide resulted in the formation of 2,5-dimethyl-1,5-hexanediol (14). 2,5-Dimethyl-5-hydroxy-1-hexanoic acid was isolated as the sodium salt by hydrolysis of the α,δ,δ -trimethyl- δ -valerolactone with sodium hydroxide with one equivalent of sodium hydroxide (15).

METHODS

Twelve Fischer 344 male rats weighing 231 ± 10 g were randomly divided into two groups (8 treated, 6 control). Doses (0.8 g/kg) were administered by gavage on an every other day regimen for two weeks. Feed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) and water were provided ad libitum and animals were weighed daily.

Following the 14 day exposure period, the rats were sacrificed by halothane overdose and the kidneys were excised 24 hours following the final dose. Histopathologic examination was performed on paraffin embedded kidney sections stained with hematoxylin and eosin. Tissues from treated rats were compared to controls for characteristic lesions of hydrocarbon-induced nephropathy, including hyaline droplet formation, tubular cysts and papillary calcification. Lesions were graded by pathologists for degree of severity.

During the first 48 hours of the initial dosing period, the rats were placed in metabolism cages and the urine collected. A 5.0 mL aliquot of each urine sample was adjusted to a pH of 4.0 and 0.2 mL glucuronidase/sulfatase (Calbiochem, La Jolla, CA) was added. The sample was shaken for 16 hours at 37°C , then cooled to room temperature and filtered through a diatomaceous earth column (Clin Elut, Analytichem International, Harbor City, CA) using methylene chloride as the eluent.

The methylene chloride extracts of the hydrolyzed rat urine were analyzed on a gas-liquid chromatograph (GC) equipped with a flame ionization detector (model 5880A, Hewlett-Packard Corp., Avondale, PA). A 25 m x 0.2 mm I.D. carbowax 20 M fused silica column Hewlett-Packard Corp., Avondale, PA). was used with injection port and detector temperatures of 200°C and 250°C , respectively. The oven temperature was programmed to rise from 60°C to 170°C at a rate of $50^{\circ}\text{C}/\text{min}$ and helium was used as the carrier gas. Additional metabolite identification was accomplished using a Hewlett-Packard 5985 gas chromatography/mass spectrometer (GC/MS) system. The GC was equipped with the identical column as the gas chromatograph above while the injection port temperature and the oven temperature were the same as previously reported. Helium was the carrier gas. The MS was a

quadrupole instrument operated in the electron impact mode with a voltage of 70eV and an ion source of 200° C.

The difficulty in totally separating all the products (including control metabolites) in the rat urine made quantitation of the metabolites impossible. However, relative areas under the peaks, listed in Table 1, were obtained from the GC program.

RESULTS

The rat urine samples were hydrolyzed with glucuronidase/sulfatase and elutants were individually analyzed by GC and GC/MS for the identification of the volatile urinary metabolites. A representative GC chromatograph is shown in Figure 1. Identified 2,5-DMH urinary metabolites along with their relative abundancies are listed in Table 1. GC analysis of rat urines not treated with glucuronidase/sulfatase showed no trace of 2,5-DMH metabolites.

Histopathologic results indicated that 2,5-DMH graded out on a level of 3 (based on a scale of 0 to 4 where 0 indicates no damage and 4 indicates severe damage) for its ability to induce hyaline droplet formation. A grade of 3 suggests moderate damage to the proximal tubule. n-Octane graded out at a level of 0 whereas 2,2,4- and 2,3,4-TMP produced severe damage which ranked at a level of 4. In one of the rats dosed with 2,5-DMH there was cast formation. No hyaline droplet formation was noted in the control animals.

DISCUSSION

The GC tracings of the 2,5-DMH dosed animals revealed the following urinary metabolites (relative abundancies) 2,5-dimethyl-1-hexanoic acid (1.0), 2,5-dimethyl-1,2-hexanediol (2.6), 2,5-dimethyl-1,5-hexanediol (5.7), 2,5-dimethyl-5-hydroxy-1-hexanoic acid (7.5) and 2,5-dimethyl-2,5-hexanediol (35.2). The relative abundancies were determined by areas of GC tracing, using tridecane as an internal standard, and assuming relative equal detection efficiencies.

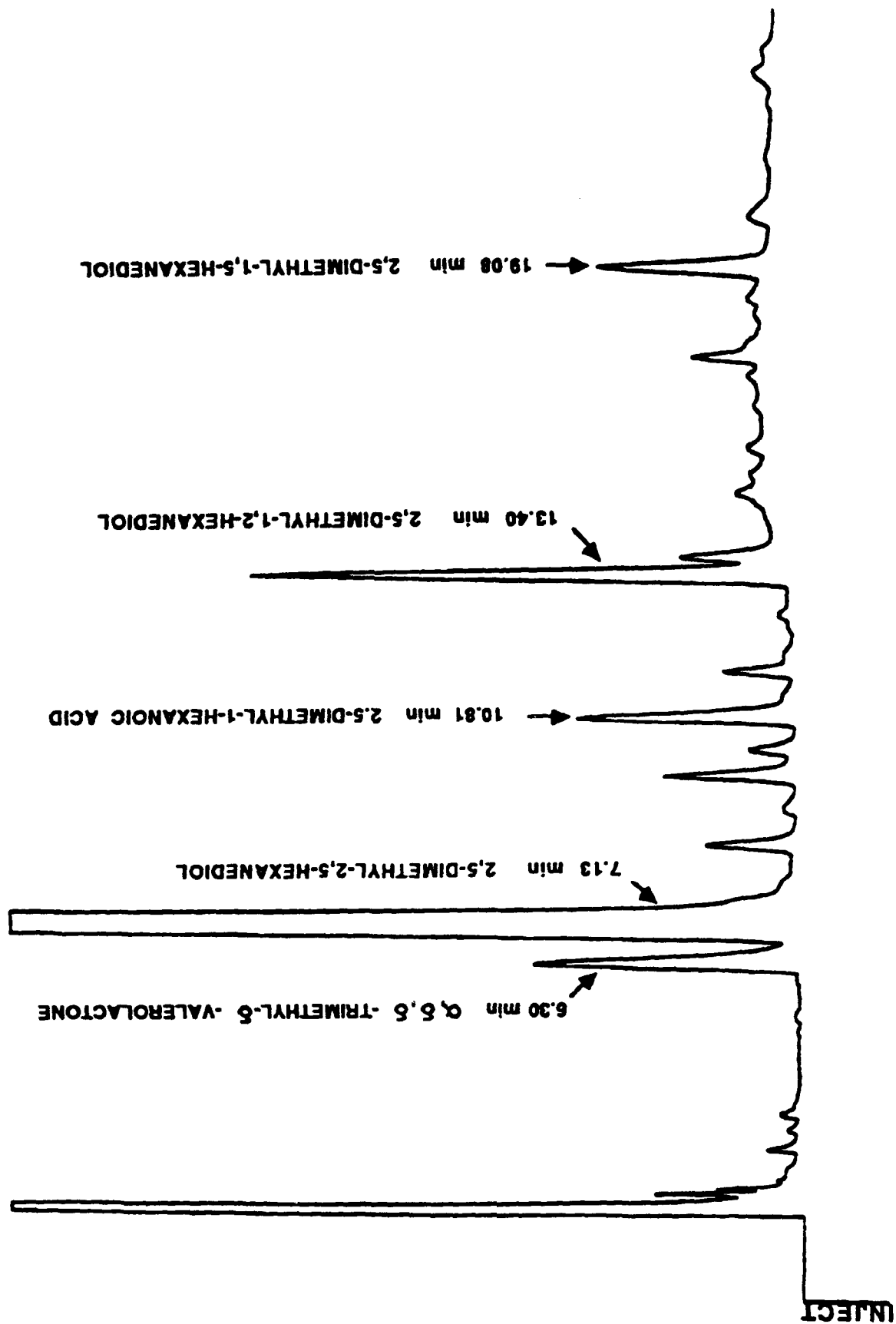
TABLE 1. 2,5-DIMETHYLHEXANE METABOLITES

ISOLATED FROM THE URINE OF FISCHER 344 MALE RATS



METABOLITE	R			GC TRACING ABUNDANCE
	R ₁	R ₂	R ₃	
2,5-Dimethyl-5-hydroxy-1-hexanoic acid	CO ₂ H	H	OH	7.5
2,5-Dimethyl-2,5-hexanediol	CH ₃	OH	OH	35.2
2,5-Dimethyl-1-hexanoic acid	CO ₂ H	H	H	1.0
2,5-Dimethyl-1,2-hexanediol	CH ₂ OH	OH	H	2.6
2,5-Dimethyl-1,5-hexanediol	CH ₂ OH	H	OH	5.7

Figure 1. Gas Chromatographic Tracing of Urine From Male Fischer 344 Rats Dosed With 2,5-Dimethylhexane



2,5-Dimethyl-5-hydroxy-1-hexanoic acid was not directly detected on GC or GC/MS, but rather as the lactone α,δ,δ -trimethyl- δ -valerolactone. The identity of the lactone was confirmed by matching the Mass Spectrum fragmentation pattern of the urinary metabolite with that of a synthesized sample of the lactone. It has been shown that hydroxy acids in which the functional groups are separated by 4 or 5 carbons, readily undergo cyclization to the lactone under the conditions of the elevated injection port temperatures of the GC (16). To confirm that the hydroxy acid was present, known samples of the lactone, the hydroxy acid and the urine sample were spotted on a thin layer chromatography (tlc) plate and eluted with ethyl acetate. The R_f of the known hydroxy acid matched that of the R_f of a metabolite in the urine sample. No tlc spot in the urine sample matched the R_f of the lactone.

A comparison of the metabolic pathways of 2,5-DMH to those of 2,2,4- and 2,3,4-TMP revealed that the major urinary metabolites found for 2,2,4- and 2,3,4-TMP were hydroxy carboxylic acids and mono-substituted carboxylic acids, while minor products were mono-substituted alcohols. There were no diols isolated. In the case of 2,5-DMH the major metabolic pathway favored the formation of diols with smaller quantities of carboxylic acids being produced. The urine of the 2,5-DMH treated rats was checked for any trace of all the possible mono-alcohol derivatives of the hydrocarbon. The results were all negative. The urinary metabolic products from all three of the aforementioned hydrocarbons seem to indicate that the formation of a δ -hydroxycarboxylic acid is a favored metabolic pathway when the penultimate carbons are substituted with methyl groups. However, a slight modification in the carbon skeleton of the hydrocarbon does significantly alter the metabolic pathway with respect to the variety of functional groups present on the metabolites.

Although 2,5-DMH did produce moderate kidney damage, it was noticeably less nephrotoxic than 2,2,4- and 2,3,4-TMP in producing the hyaline droplet nephropathy. A possible explanation as to the decreased nephrotoxicity is the larger proportion of 2,5-DMH which was metabolized into the diol. Neither 2,2,4- nor 2,3,4-TMP yielded any urinary diols. A recent report showed that not all chemicals which bind to α_2 u-globulin will slow the degradation of the protein (17). To illustrate the importance of diol formation, d-Limonene, which is metabolized to both d-limonene-

1,2-oxide and d-limonene-8,9-oxide. The 8,9-oxide rapidly underwent hydrolysis to the d-limonene-8,9-diol which did not bind to the α_2 -globulin. The d-limonene-1,2-oxide with a longer lifetime resist hydrolysis and is found to bind to the α_2 -globulin and produce the hydrocarbon nephropathy (18). There are no reported examples of diols binding to α_2 -globulin.

In summary, 2,5-DMH, when metabolized, yielded derivatives which are capable of producing a hyaline droplet type of nephropathy at a diminished level from the previously evaluated nephrotoxic hydrocarbons 2,2,4- and 2,3,4-TMP. Diols, which have not been shown to bind with α_2 -globulin, were the principal urinary metabolites of 2,5-DMH and this may account for the reduced nephrotoxicity.

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THE METABOLISM OF 2-METHYLHEPTANE IN MALE FISCHER 344 RATS

M. Paul Serve^a, D.D. Bombick^a, J. Matthew Clemens^a,
Gayle A. McDonald^a and David R. Mattie^b

^a Department of Chemistry, Wright State University, Dayton, OH 45435

^b Toxicology Division, Occupational and Environmental Health Directorate,
Armstrong Laboratory, Wright-Patterson AFB, OH 45433-6573.

ABSTRACT

The urinary metabolites of 2-methylheptane (2-MH) in male Fischer 344 rats, administered the hydrocarbon by gavage, included 2-methyl-1,2-heptanediol, 2-methyl-1,5-heptanediol, 2-methyl-2,5-heptanediol, 2-methylheptanoic acid and 2-methyl-5-hydroxy-1-heptanoic acid. Metabolism strongly favored the formation of diols. The metabolites were identified using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Histopathologic examination of the kidneys revealed minimal hyaline droplet formation (α_2 -globulin nephropathy) in the proximal tubule area.

INTRODUCTION

2-Methylheptane (2-MH), an isomer of octane is a component of crude oil and a product of gasoline combustion engines [1,2]. It is not surprising, therefore, that 2-MH has been found as a component of the air pollution of major cities [3,4]. As a constituent of tobacco smoke, 2-MH is frequently passively taken into the body [5,6]. 2-MH has been identified as a volatile chemical emitted from plastic building materials [7] and as a trace compound in landfill gas [8]. Isomers of octane, when injected into animals, have been shown to produce hemorrhage, edema and poly-

morphonuclear leukocytic reactions such as angitis, abscess formation, thrombosis and fibrosis [9].

Certain branched chain isomers of octane e.g. 2,2,4-trimethylpentane (2,2,4-TMP) and 2,3,4-trimethylpentane (2,3,4-TMP) have been reported to induce a proximal tubular nephropathy in male rats [10,11]. This nephropathy is characterized by the inability of the rat to breakdown the testosterone controlled protein α_2 -globulin through the normal lysosomal enzyme processes [12]. 2-MH, similar to other hydrocarbons, is a totally non-polar molecule. Hydrocarbons once in the animal body, are normally gotten rid of by either exhalation or excretion via the urine. The latter process requires metabolic conversion of the hydrocarbon into a water soluble derivative. It is the production of metabolites with certain functionalities, which, by tying up the α_2 -globulin and inhibiting its degradation, is generally considered to be responsible for the hydrocarbon-induced nephropathy cited above.

Because of its ubiquitous nature in the environment and since many people will be exposed to various concentrations of 2-MH, it was decided to examine the metabolic fate of 2-MH in male rats. Male rats are frequently used to study the metabolism of xenobiotic materials because of the similarity of biotransformation reactions that exist between man and rat. Secondly, a comparison of the ability of 2-MH to 2,2,4- and 2,3,4-TMP to induce the male rat nephropathy would yield interesting information regarding the extent and positioning of structural branching necessary to induce the nephrotoxicity.

MATERIALS

2-Methyl-1-heptene, 6-methyl-1-heptene, 6-methyl-6-hepten-3-ol, and 2-ethylcyclopentanone were purchased from Wiley Organics, Coshcoton, OH.

2-Methyl-1,2-heptanediol and 6-methyl-1,2-heptanediol were prepared by the reaction of 2-methyl-1-heptene and 6-methyl-1-heptene, respectively with osmium tetroxide [13]. 2-Methyl-1,5-heptanediol was synthesized by the hydroboration of 6-methyl-6-hepten-3-ol [14]. 2-Methyl-2,5-heptanediol was obtained via the mercuric acetate-sodium borohydride reaction of 6-methyl-6-hepten-3-ol [15]. 2-Methylheptanoic

acid was prepared using literature [16]. Treatment of 2-ethyl-5-methylcyclopentanone, obtained from the alkylation of the pyrrolidine enamine of 2-ethylcyclopentanone, with trifluoroacetic acid gave 2-methyl-5-heptanolactone [17].

METHODS

Twelve Fischer 344 male rats weighing 237 ± 16 grams were randomly divided into two groups (8 treated, 4 control). Dose (0.8 g/kg) of 2-MH were administered by gavage on an every other day regimen for two weeks. Feed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) and water were provided ad libitum and animals were weighed daily.

Following the 14 day exposure period, the rats were sacrificed by halothane overdose and the kidneys were excised 24 hours following the final dose. Histopathologic examination was performed on paraffin embedded kidney sections stained with hematoxylin and eosin. Tissues from treated rats were compared to controls for characteristic lesions of hydrocarbon-induced nephropathy, including hyaline droplet formation, tubular cysts and papillary calcification. Lesions were graded by pathologists for degree of severity.

During the first 48 hours of the initial dosing period, the rats were placed in metabolism cages and the urine collected. A 5.0 mL aliquot of each urine sample was adjusted to a pH of 4.0 and 0.2 mL glucuronidase/sulfatase (Calbiochem, La Jolla, CA) was added. The sample was shaken for 16 hours at 37°C, then cooled to room temperature and filtered through a diatomaceous earth column (Clin Elut, Analytichem International Harbor, CA) using methylene chloride as the eluent.

The methylene chloride extracts of the hydrolyzed rat urine were analyzed on a gas-liquid chromatograph (GC) equipped with a flame ionization detector (model 3500, Varian Corp., Walnut Creek CA). A 30 m x 0.32 mm I.D. Carbowax 20M on polyethylene glycol column Alltech Associates Inc., Deerfield IL) with injection port and detection temperatures of 250°C. The oven temperature was programmed to rise from 100°C to 200°C at a rate of 5°C/min and helium was used as the carrier gas. Additional metabolite identification was accomplished using a Finnegan MAT INCOS 50 -Varian 3400 gas chromatography/mass

spectrometer (GC/MS) system (Varian Corp., Walnut Creek, CA). The GC was equipped with identical column as the gas chromatograph above while the injection port and the oven temperatures were the same as previously reported. Helium was the carrier gas. The MS was a quadrupole instrument operated in the electron impact mode with a voltage of 70eV and an ion source of 200° C.

The difficulty in totally separating all the products (including control metabolites) in the rat urine made quantitation of the metabolites impossible. However, relative areas under the peaks, listed in Table 1 were obtained from the GC program.

RESULTS

The rat urine samples were hydrolyzed with glucuronidase/sulfatase and elutants were individually analyzed by GC and GC/MS for the identification of the volatile urinary metabolites. A representative GC chromatogram is shown in Figure 1. GC analysis of rat urines not treated with glucuronidase/sulfatase showed no trace of 2-MH metabolites.

Histopathologic results indicated that 2-MH graded out on a level of 1 (based on a scale of 0 to 4 where 0 indicated no damage and 4 indicated severe damage). A grade of 1 suggests minimal damage to the proximal tubule. n-Octane graded out at a level of 0 whereas 2,5-dimethylhexane (2,5-DMH), another isomer of octane produced nephrotoxic damage rated at a level of 3 [18]. 2,2,4- and 2,3,4-TMP produced severe damage to the male rat proximal tubule which graded out to a level of 4. In none of the rats dosed with 2-MH was there any indication of cast formation. There was no trace of any 2-MH metabolites extracted from the homogenized kidneys of the dosed rats. No hyaline droplet formation was noted in the control animals.

Figure 1. GAS CHROMATOGRAPHIC TRACING OF URINE FROM MALE
FISCHER 344 RATS DOSED WITH 2-METHYLHEPTANE

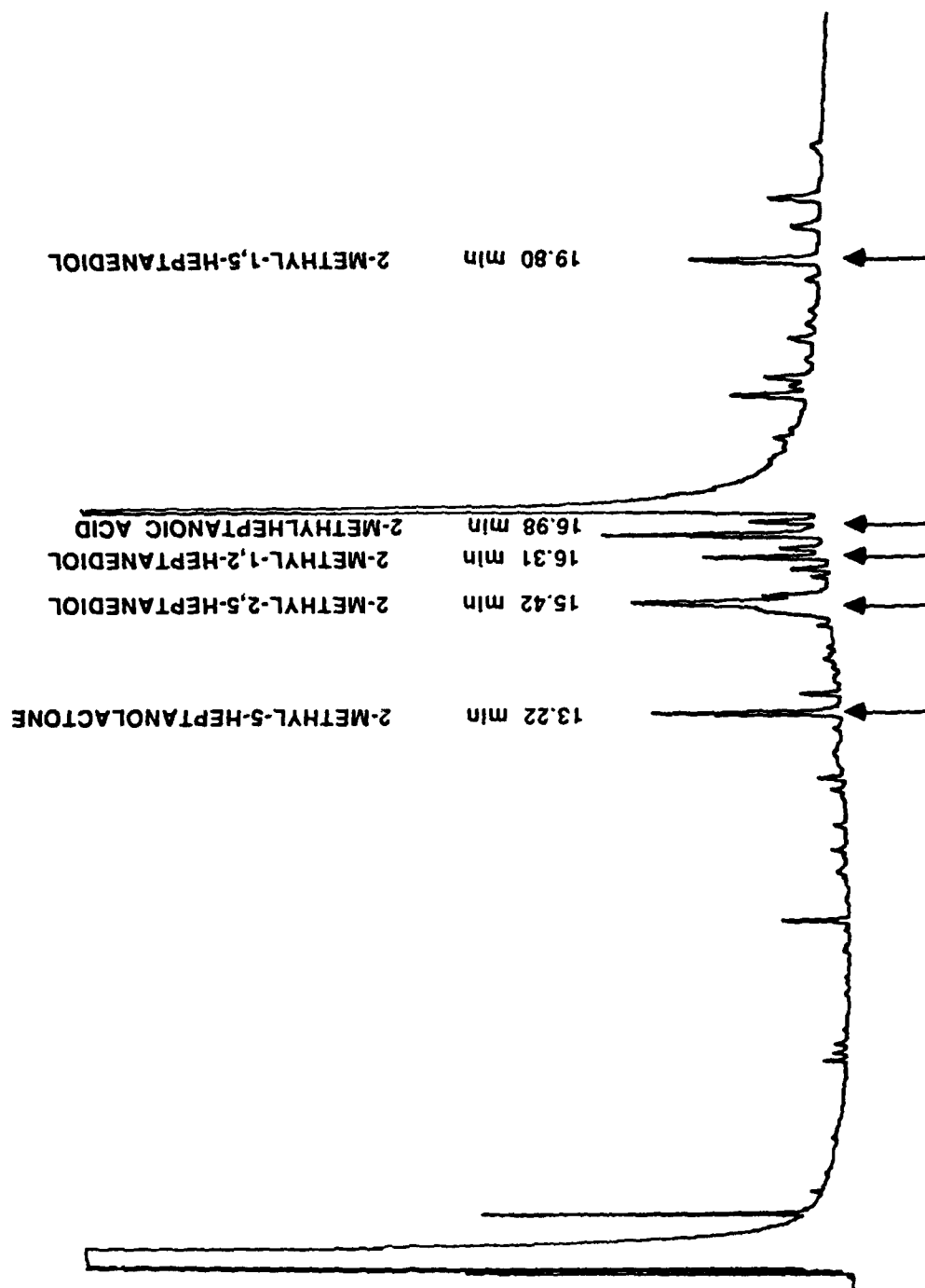
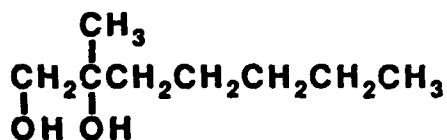
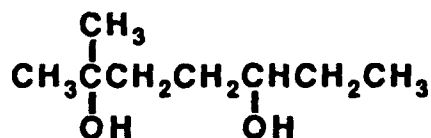


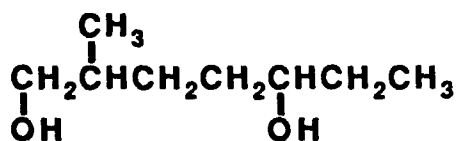
TABLE 1. METABOLITES OF 2-METHYLHEPTANE



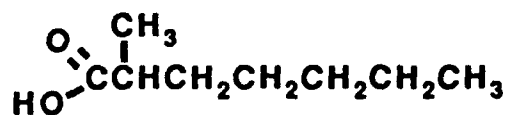
2-METHYL-1,2-HEPTANEDIOL
(1.25)



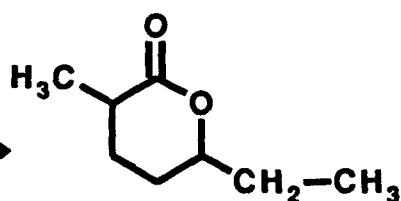
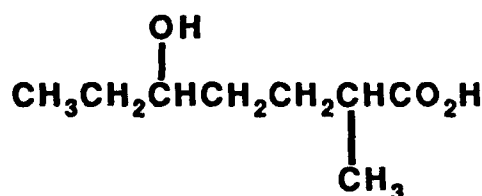
2-METHYL-2,5-HEPTANEDIOL
(1.8)



2-METHYL-1,5-HEPTANEDIOL
(1.0)



2-METHYLHEPTANOIC
ACID (1.4)



2-METHYL-5-HEPTANOLACTONE
(1.6)

The GC tracings of the animals dosed with 2-MH established the presence of the following urinary metabolites (relative abundancies) 2-methyl-1,5-heptanediol (1.0), 2-methyl-1,2-heptanediol (1.25), 2-methylheptanoic acid (1.4) 2-methyl-5-heptanolactone (1.6) and 2-methyl-2,5-heptanediol (1.8). The relative abundancies were determined by integrating the areas of GC tracings, using tridecane as an internal reference standard, and assuming relative equal detection efficiencies. The

structures of the urinary metabolites of 2-MH are presented in Table 1. The metabolite 2-methyl-5-heptanolactone was not a true metabolite, but was the result of cyclization of 2-methyl-5-hydroxy-1-heptanoic acid under the elevated injection port temperature conditions of the GC. It has been previously shown that lactone formation of hydroxy acids, in which the hydroxy group and the acid function are separated by 4 or 5 carbons, readily occurs. The identification of the lactone structure was accomplished by examining the MS fragmentation pattern of the lactone. A major MS fragmentation peak of the lactone corresponded to the molecular ion - C_2H_5 . A characteristic fragmentation pattern of δ -lactones is the loss of the alkyl group attached to the δ -carbon [19]. In addition, MS fragmentation peaks at $m/z=56$ and 70 were also indicative of a δ -lactone. To confirm the presence of the 2-methyl-5-ethylcyclopentanone was oxidized to a mixture of 2-methyl-5-heptanolactone and 2-ethyl-5-hexanolactone via the Baeyer-Villiger reaction. Known samples of the hydroxy acid, the lactone and the urine were subjected to thin layer chromatography (tlc) using ethyl acetate as the eluent. The R_f of the known hydroxy acid matched the R_f of a metabolite in the urine sample. No TLC spot in the urine sample matched the R_f of the lactone.

Comparing the metabolic pathways of 2-MH to those of other branched chain acyclic hydrocarbons reveals similarities and differences in the structure and abundancies of the various types of metabolites isolated. 2-MH akin to 2,5-dimethylhexane (2,5-DMH) was metabolized to diols, which were positioned at the 1,2- 1,5- and the 2,5-sites [19]. In addition both 2-MH and 2,5-DMH yielded the corresponding 2-alkyl alkanolic acids and the 2-alkyl-5-hydroxy-alkanoic acids. In neither case was there any trace of an alcohol. A difference in the metabolism of 2-MH and 2,5-DMH was in the relative abundancies of the urinary metabolites found. In the instance of 2-MH the metabolites were isolated in almost equal amounts, while for 2,5-DMH, there was a wide range in the quantities of metabolites produced. The 2,5-dimethyl-2,5-hexanediol and the 2,5-dimethyl-5-hydroxy-1-hexanoic acid were found in abundancies 38.7 and 8.3 times the amount of 2,5-dimethyl-1-hexanoic acid, the least abundantly found metabolite. The highly branched chain hydrocarbons 2,2,4- and 2,3,4-TMP were metabolized to monoalcohols, carboxylic acids and 5-hydroxy-carboxylic acids; there was no trace of diol formation.

Unlike n-octane, which yielded urinary metabolites containing less than 8 carbons, there was no vestige of any urinary metabolites of 2-MH in which carbon atoms had been lost [20].

In conclusion, limiting the substitution of an octane isomer to a methyl group at the 2-position, changes the metabolism from that of n-octane. The structure of the urinary metabolites of 2-MH appear to resemble the metabolites of 2,5-DMH, the only difference being in the relative amounts of the metabolites formed. In the ability to induce renal proximal tubular damage to male rats, 2-MH more closely resembles n-octane than 2,5-DMH. This would lead to the conclusion that the more closely related in structure an octane molecule is to n-octane, the less the ability of the metabolites to interfere with α_2 -globulin degradation and produce the hydrocarbon nephrotoxicity.

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THE METABOLISM OF 3-METHYLHEPTANE IN MALE FISCHER 344 RATS

M. Paul Serve^a, David D. Bombick^a, J. Matthew Clemens^a, Gayle A. McDonald^a and David R. Mattie^b

^a Department of Chemistry, Wright State University, Dayton, OH 45435

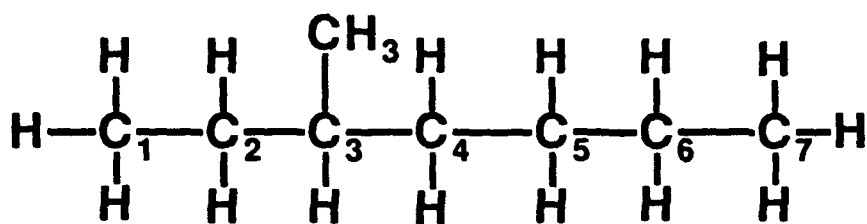
^b Toxic Hazards Division, Armstrong Laboratory, Wright-Patterson AFB, OH 45433.

ABSTRACT

3-Methylheptane (3-MH) was administered by gavage to male Fischer 344 rats. 48 Hour urines were collected, processed and the following urinary metabolites identified by gas chromatography and gas chromatography/mass spectrometry: 3,5-diethyl-2,3-dihydrofuran, 3-ethyl-6-methyl-2,3-dihdropyran, 3-methyl-3-heptanol, 3-methyl-2-heptanol, 5-methyl-2-heptanol, 2-n-butyl-1,3-butanediol, 2-ethyl-1,3-hexanediol, β -methyl- δ -enantholactone, 3-methyl-3,4-heptanediol, 3-methyl-2,3-heptanediol, 3-methyl-3,5-heptanediol, 2-ethyl-1,4-hexanediol, δ -methyl- δ -enantholactone, 5-methyl-2,5-heptanediol, 2-ethyl-1,5-hexanediol and 2-ethylhexanoic acid (2-EHA). One of the most interesting metabolites was 2-EHA, a known peroxisome proliferator and a metabolite of di(2-ethylhexyl)phthalate, a known renal tumor promoter. Histopathological results indicated that unlike 2-methylheptane, 3-MH was a more potent inducer of the well-known α_2 -globulin nephropathy.

INTRODUCTION

3-Methylheptane (3-MH) is a commonly found component of solvents [1] and fuels [2]. As a naphtha component of crude oils [3], it is not surprising that 3-MH has been isolated from exhaust gas, from in-use passenger autos [4, 5] and is found in the air of various cities [6]. The pervasiveness of 3-MH in the environment is reflected by the detection of the hydrocarbon as an air pollutant from activated sludge wastewater treatment plants [7], as a component of landfill gas [8] and as a soil contaminant next to airplane tarmacs [9]. Because of the ubiquitous nature of 3-MH in the air, the hydrocarbon has been studied for its ability to initiate photochemical smog [10, 11, 12, 13].



3-METHYLHEPTANE

Recently a derivative of 3-MH, namely 2-ethylhexanoic acid (2-EHA) was reported to be a peroxisome and mitochondrial proliferator in mouse liver [14]. 2-EHA was later demonstrated to induce the production of cytosolic and microsomal epoxide hydrase in mouse liver [15, 16]. It is well established that peroxisome proliferators produce hepatomegaly and are potent cancerous tumor inducers in mice and rats [17]. Information on the effects of peroxisome proliferators with the kidney is scanty. Although peroxisome proliferators do react with the P1 and P2 portions of the renal proximal tubule, no kidney enlargement or renal proliferation of the endoplasmic reticulum has been observed in experimental animals. The principal effects of peroxisome proliferators on the kidney have been an increase in peroxisomal β -oxidation enzymes and induction of the polypeptide PPA 80,000 [18]. Although peroxisome proliferators have not been shown to be renal tumor inducers, Kurkokawa has reported that the peroxisome proliferator di(2-ethylhexyl)phthalate (DEHP) was able to function as a renal tumor promoter after induction by N-ethyl-N-hydroxy-ethylnitrosamine [19]. Since DEHP was administered orally, a possible metabolite of DEHP would be 2-EHA.

It is well established that branched chain hydrocarbon isomers of octane e.g. 2,2,4- and 2,3,4-trimethylpentane induce a proximal tubular nephropathy in male rats characterized by hyaline droplet formation followed by cellular necrosis and then cellular regeneration [20, 21]. The hyaline droplets which accumulate have been shown to contain $\alpha_2\text{u}$ -globulin ($\alpha_2\text{u}$), a low molecular protein synthesized only in the male rat [22]. The nephrotoxicity of hydrocarbons is believed to be due to the formation of metabolites, which contain reactive functional groups and bind to $\alpha_2\text{u}$. These metabolites- $\alpha_2\text{u}$ complexes interfere with cellular

biochemical reactions, including the breakdown of $\alpha_2\text{u}$, which can cause serious cellular damage, including cell death. A possible explanation for the tumorigenicity of the branched chain hydrocarbons is that the extensive kidney cell death caused by metabolites of the hydrocarbons initiates a rapid cell proliferation to repair the damage. The rapid cell proliferation increases the opportunities for mutations that can lead cells to become tumorous [23]. Another possible explanation of the hydrocarbon-induced nephrotoxicity is that certain metabolites may function as peroxisome proliferator and induce the formation of various enzymes, including those involved in the synthesis of $\alpha_2\text{u}$.

The ubiquitousness of 3-MH in the environment and its structural similarity to other branched chain octane isomers stimulated our interest in evaluating the metabolism of 3-MH in rats and comparing the nephrotoxicity of the molecule to other branched chain hydrocarbons. Previous studies on the isomers of octane have indicated that functional groups such as alcohols, acids and diols have been incorporated into the hydrocarbon structure [20, 24]. Similar metabolic pathways for 3-MH could yield 2-EHA as a metabolite. Since 2-EHA has already been established as peroxisome proliferator in rats, 3-MH could prove to be one of the more nephrotoxic branched chain octanes. This would lend credence to the proposal that peroxisome proliferators play a major role in explaining the hydrocarbon induced nephrotoxicity.

MATERIALS

3-Methyl-1-heptanol, 3-methyl-2-heptanol, 3-methyl-3-heptanol, 3-methyl-4-heptanol, 5-methyl-1-heptanol, 5-methyl-2-heptanol, 5-methyl-3-heptanol, 2-ethyl-1-hexanol, 2-ethylhexanoic acid, 2-ethyl-1,3-hexanediol, 1,2,3-trimethylcyclopentene, 5-methyl-5-hydroxy-2-heptene, 3-methyl-3-hydroxy-2-heptene, 2-ethylcyclopentanone, 3-methyl-1-pentene, 1-bromo-2-butene and 2-butanone were purchased from Wiley Organics, Coshocton, OH. Osmium tetroxide, trifluoroacetic anhydride, and diborane in tetrahydrofuran were purchased from Aldrich Chemical Company, Milwaukee, WI. 5-Methyl-2,5-heptanediol was prepared by the hydroboration of 5-methyl-5-hydroxy-2-heptene [25]. 3-Methyl-2,3-heptanediol was synthesized by the treatment of 3-methyl-3-hydroxy-2-heptene with mercuric acetate and sodium borohydride [26].

ϵ -Methyl-d-enanthanolactone was isolated from the reaction of 5-methyl-5-methylcyclopentanone with trifluoroacetic acid anhydride [27]. 3-Methyl-2,5-heptanediol was prepared using the literature procedure [28]. β -Methyl- δ -enantholactone and δ -methyl- δ -lactone were synthesized by the reported methodology [29]. 2-n-Butyl-1,3-butanediol was prepared according to the literature procedure [30]. 2-Ethyl-1,5-hexanediol was isolated according to the reported technique [31]. 3-Methyl-3,4-heptanediol was recovered from the reaction of methyl vinyl ketone and crotonaldehyde [32]. 3-Methyl-3,5-heptanediol synthesized according to the report of Pastureau [33]. 2,5-Dimethyl-2-ethyltetrahydrofuran and 2,3,6-trimethyltetrahydropyran were prepared by the acid catalyzed dehydration of 5-methyl-2,5-heptanediol and 3-methyl-2,6-heptanediol, respectively [34].

METHODS

Twelve Fischer 344 male rats weighing 237 $\pm$ 16 grams were randomly divided into two groups (8 treated, 4 control). Doses (0.8 g/kg) of 3-MH were administered by gavage on an every other day regimen for two weeks. Feed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) and water were provided ad libitum and animals were weighed daily. Following the 14 day exposure period, the rats were sacrificed by halothane overdose and the kidneys were excised 24 hours following the final dose. Histopathologic examination was performed on paraffin embedded kidney sections stained with hematoxylin and eosin. Tissues from treated rats were compared to controls for characteristic lesions of hydrocarbon-induced nephropathy, including hyaline droplet formation, tubular cysts and papillary calcification. Lesions were graded by pathologists for degree of severity.

During the first 48 hours of the initial dosing period, the rats were placed in metabolism cages and the urine collected. A 5.0 mL aliquot of each urine sample was adjusted to a pH of 4.0 and 0.2 mL glucuronidase/sulfatase (Calbiochem, La Jolla, CA) was added. The sample was shaken for 16 hours at 37°C, then cooled to room temperature and filtered through a diatomaceous earth column (Clin Elut, Analytichem International Harbor, CA) using methylene chloride as the eluent.

The methylene chloride extracts of the hydrolyzed rat urine were analyzed on a gas-liquid chromatograph (GC) equipped with a flame ionization detector (model 3500, Varian Corp., Walnut Creek CA). A 30 m x 0.32 mm I.D. Carbowax 20M on polyethylene glycol column Alltech Associates Inc., Deerfield IL) with injection port and detection temperatures of 250° C. The oven temperature was programmed to rise from 100° C to 200° C at a rate of 5° C/min and helium was used as the carrier gas. Additional metabolite identification was accomplished using a Finnegan MAT INCOS 50 -Varian 3400 gas chromatography/mass spectrometer (GC/MS) system (Varian Corp., Walnut Creek, CA). The GC was equipped with an identical column as the gas chromatograph above while the injection port and the oven temperatures were the same as previously reported. Helium was the carrier gas. The MS was a quadrupole instrument operated in the electron impact mode with a voltage of 70eV and an ion source of 200° C.

The difficulty in totally separating all the products (including control metabolites) in the rat urine made quantitation of the metabolites impossible. However, relative areas under the peaks, listed in Table 1 were obtained from the GC program.

RESULTS AND DISCUSSION

The individualized rat urine samples, upon hydrolysis with glucuronidase/sulfatase were analyzed by GC and GC/MS for the identification of volatile urinary metabolites. A representative GC chromatogram is shown in Figure 1. The metabolites names and structures, which correspond to the numbers in the GC chromatogram are presented in Tables 1 and 2, respectively. Although the metabolites numbered 1, 2, 8 and 13 are those identified by the GC and GC/MS, they are not the true structures found in the urines. Dihydrofurans and dihydropyrans have previously been isolated when appropriately substituted ketoalcohols were subjected to the high temperatures found in the injection port of the GC [35]. Likewise lactone metabolites have been shown to result from the high temperature injection port cyclization of suitably substituted hydroxyacids [36]. Thus, for the purposes of clarification, metabolites 1, 2, 8 and 13 are represented in Table 2 by the acyclic structure of the appropriate precursor molecule. GC chromatograms

of 3-MH dosed rat urines, not treated with glucuronidase/sulfatase, showed no trace of 3-MH metabolites. From Table 2, it was perceived that every carbon of the 3-MH molecule, in at least two metabolites, was subjected to metabolic oxidation. The position on the 3-MH where the metabolic oxidation occurred to the greatest extent was the 3-methyl substituent. There were seven individual metabolites (1, 2, 6, 7, 12, 15 and 16) in which the 3-methyl group had suffered at least monooxidation. With the wide number of metabolites in which the 3-methyl group had been converted to a CH_2OH group, it was not surprising that 2-EHA was isolated as a metabolite. Although metabolic conversion of the terminal carbon to a carboxylic acid has been previously found for other branched and unbranched acyclic hydrocarbons [24, 36], this is the first report of a substituted CH_3 group on a long chain hydrocarbon being converted to a CO_2H . The C-H bond at carbon-3 was also especially vulnerable to oxidation with appearance of six separate metabolites showing hydroxylation at carbon-3.

Further examination of Table 2 revealed that the monoalcohol metabolites underwent metabolism at either the penultimate carbon or at carbon-3. Additionally, with the exception of metabolite 12, each of the diol metabolites had a hydroxy group substituted at either carbon-2, carbon-3 or carbon-6 of 3-MH. Metabolite 13 also had an OH substituted at carbon-3. The only metabolic products which show no oxidation at carbon-2, carbon-3 or carbon-6 of 3-MH were 1, 2, 8, 12 and 16. However, metabolites 1, 2, 12 and 16 all show oxidation occurring at the 3-methyl substituent. Finally, it was noted that simple monooxidation of the 3-methyl substituent was not sufficient for urinary excretion of 3-MH from the rat. There was no trace of 2-ethylhexanol isolated from any of the individual rat urines.

A possible metabolic sequence of events to explain the formation of the various metabolites is shown in Figure 2 (Part A and B).

FIGURE 1. METABOLITES ISOLATED FROM THE URINES OF MALE FISCHER
344 RATS DOSED WITH 3-METHYLHEPTANE

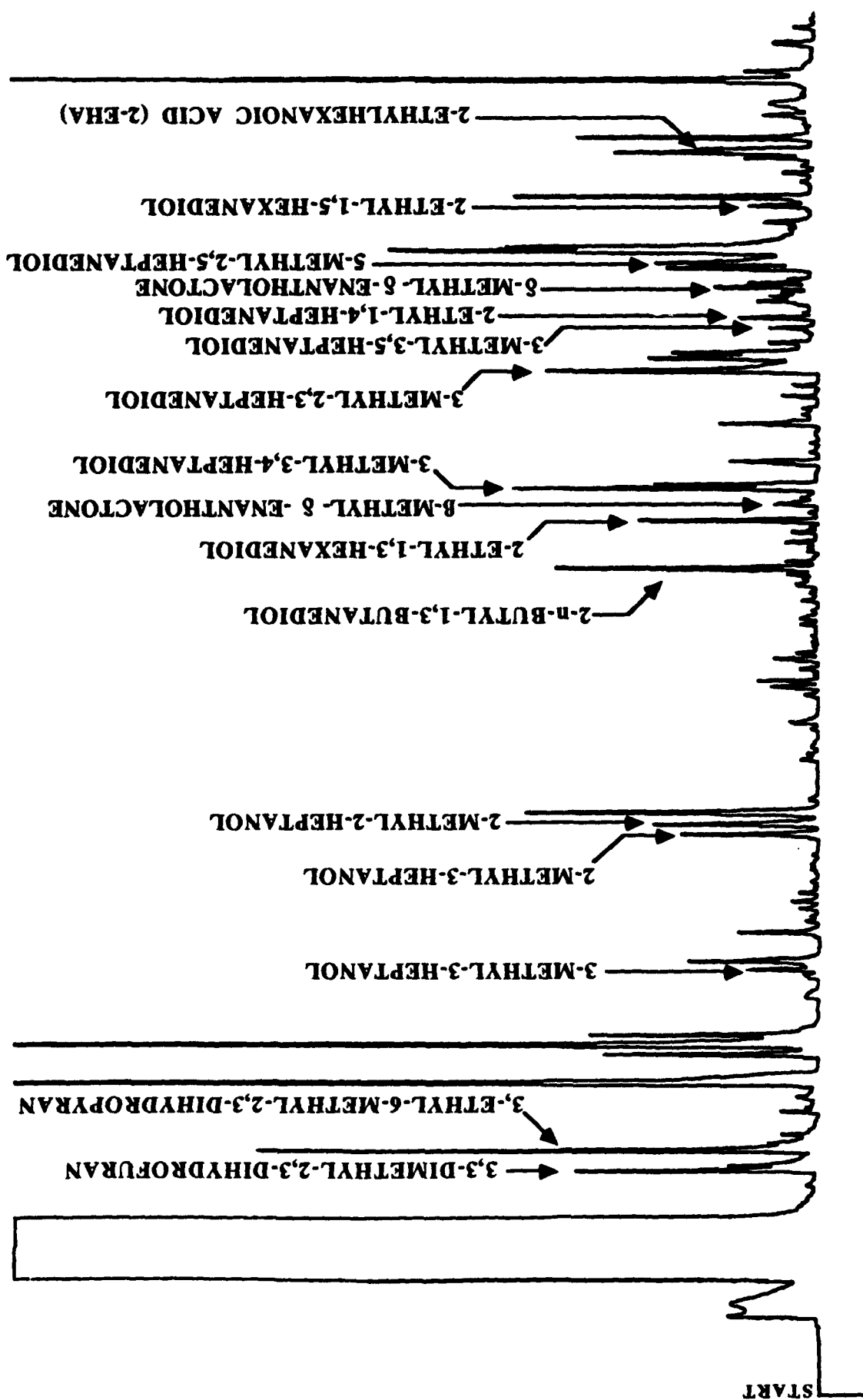
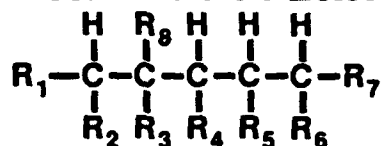


TABLE 1. METABOLITES ISOLATED FROM THE URINES OF MALE FISCHER
344 RATS DOSED WITH 3-METHYLHEPTANE

METABOLITE	RELATIVE ABUNDANCE
1. 3,5-DIETHYL-2,3-DIHYDROFURAN	4.3
2. 3-ETHYL-6-METHYL-2,3-DIHYDROPYRAN	9.9
3. 3-METHYL-3-HEPTANOL	1.5
4. 3-METHYL-2-HEPTANOL	3.9
5. 5-METHYL-2-HEPTANOL	7.4
6. 2-n-BUTYL-1,3-BUTANEDIOL	2.8
7. 2-ETHYL-1,3-HEXANEDIOL	5.5
8. β -METHYL- δ -ENANTHOLACTONE	1.5
9. 3-METHYL-3,4-HEPTANEDIOL	8.0
10. 3-METHYL-2,3-HEPTANEDIOL	7.9
11. 3-METHYL-3,5-HEPTANEDIOL	1.3
12. 2-ETHYL-1,4-HEXANEDIOL	1.6
13. δ -METHYL- δ -ENANTHOLACTONE	2.6
14. 5-METHYL-2,5-HEPTANEDIOL	3.0
15. 2-ETHYL-1,5-HEXANEDIOL	1.0
16. 2-ETHYLHEXANOIC ACID (2-EHA)	2.5

TABLE 2 ACYCLIC DERIVATIVES ISOLATED FROM THE URINES OF RATS
DOSED WITH 3-METHYLHEPTANE



METABOLITE PEAK NUMBER	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈
1	CH ₃	H	H	H	=O	H	CH ₃	CH ₂ OH
2	CH ₃	H	H	H	H	=O	CH ₃	CH ₂ OH
3	CH ₃	H	OH	H	H	H	CH ₃	CH ₃
4	CH ₃	OH	H	H	H	H	CH ₃	CH ₃
5	CH ₃	H	H	H	H	OH	CH ₃	CH ₃
6	CH ₃	OH	H	H	H	H	CH ₃	CH ₂ OH
7	CH ₃	H	H	OH	H	H	CH ₃	CH ₂ OH
8	CO ₂ H	H	H	H	OH	H	CH ₃	CH ₃
9	CH ₃	H	OH	OH	H	H	CH ₃	CH ₃
10	CH ₃	OH	OH	H	H	H	CH ₃	CH ₃
11	CH ₃	H	OH	H	OH	H	CH ₃	CH ₃
12	CH ₃	H	H	H	OH	H	CH ₃	CH ₂ OH
13	CH ₃	H	OH	H	H	H	CO ₂ H	CH ₃
14	CH ₃	H	OH	H	H	OH	CH ₃	CH ₃
15	CH ₃	H	H	H	H	OH	CH ₃	CH ₂ OH
16	CH ₃	H	H	H	H	H	H	CO ₂ H

Histopathologic results indicated that 3-MH graded out on a level of 3 (based upon a scale of 0 to 4, where 0 = no damage, 1 = minimal damage, 2 = mild damage, 3 = moderate damage and 4 = severe damage). The moderate damage produced by the 3-MH was demonstrated by the extensive hyaline droplet formation. Previous studies of acyclic hydrocarbon nephrotoxicity have shown that n-octane graded out at a level of 0, 2-methylheptane (2-MH) at a level of 1 [37], while 2,5-dimethylhexane produced damage rated at a level of 3 [38]. The molecules 2,2,4- and 2,3,4-TMP occasioned damage, which graded out at a level of 4. In none of

the rats dosed with 3-MH was their evidence of cast formation. There was also no trace of any metabolite of 3-MH in the homogenized kidneys of the dosed rats. Lastly, no extra hyaline droplet formation was noted in the proximal tubules of the kidneys of the control animals.

A possible explanation as to the increased nephrotoxicity of 3-MH versus 2-MH or n-octane may lie in the finding of 2-EHA as a urinary metabolite. As stated earlier, 2-EHA is a known peroxisome proliferator. Peroxisome proliferators frequently are fatty acids and have been shown to produce serious hepatotoxicity, including tumor formation [39]. The livers of the rats treated with 3-MH were not examined for enlargement or tumor formation. In an earlier investigation concerning the ability of the metabolites of the potent nephrotoxic chemical 2,2,4-TMP to induce $\alpha_2\text{u}$ accumulation, it was determined that 2,4,4-trimethylpentanoic acid (2,4,4-TMPCOOH) caused a significant accumulation of $\alpha_2\text{u}$ [40]. However, 2,4,4-TMPCOOH was shown not to compete for binding to $\alpha_2\text{u}$ with 2,4,4-trimethyl-2-pentanol, a potent binder of $\alpha_2\text{u}$ and a strong inducer of $\alpha_2\text{u}$ [41]. Additionally, a previous study demonstrated that 2,4,4-TMPCOOH affected the liver by increasing the synthesis of $\alpha_2\text{u}$ in hepatocytes isolated from male rats [42]. It is possible that 2,4,4-TMPCOOH is a peroxisome proliferator and the increase in $\alpha_2\text{u}$ synthesis is due to some as yet unknown mechanism related to peroxisome induction enzyme synthesis. Thus, the hydrocarbon-induced nephropathy may be a combination of both the synthesis of $\alpha_2\text{u}$, induced by a metabolite, and the formation of an $\alpha_2\text{u}$ -metabolite complex, which strongly resists hydrolysis and elicits the characteristic nephrotoxic signs. The questions then (1) are metabolites, which increase $\alpha_2\text{u}$ also peroxisome proliferators; and (2) is the ability to induce the synthesis of $\alpha_2\text{u}$ a property of all peroxisome proliferators? To answer these questions, one should examine the metabolites of hydrocarbons which have been shown to be capable of inducing the $\alpha_2\text{u}$ nephrotoxicity and see if they are also peroxisome proliferators. The long term effects of peroxisome proliferators on humans have not been fully explored. If indeed it is found that hydrocarbon metabolites have the property of peroxisome proliferators and that peroxisome proliferators are potentially toxic to life, the metabolism of various hydrocarbons, which humans and other valuable animal species is a topic that must be thoroughly explored.

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THE ISOLATION AND IDENTIFICATION OF THE URINARY METABOLITES OF 4-METHYLHEPTANE IN MALE FISCHER 344 RATS

M. Paul Serve^a, D.D. Bombicka^a, J. Matthew Clemens^a, Theresa M. Rezek^a,
Gayle A. McDonald^a and David R. Mattie^b

^a Department of Chemistry, Wright State University, Dayton, OH 45435

^b Toxicology Division, Occupational and Environmental Health Directorate,
Armstrong Laboratory, Wright-Patterson AFB, OH 45433-6573.

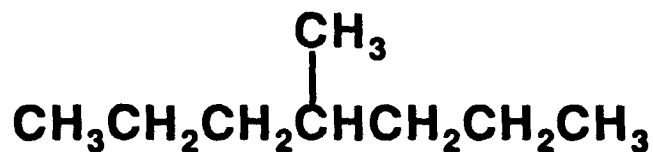
ABSTRACT

The urinary metabolites of 4-methylheptane (4-MH) in male Fischer 344 rats, administered the hydrocarbon by gavage, included 6-hydroxy-4-methyl-2-heptanone, 4-methyl-3,5-heptanediol, di-n-propyl acetic acid, 4-methyl-2,5-heptanediol, 2-methyl-3-hydroxy-1-pentanoic acid, 4-methyl-1-heptanoic acid, 4-methyl-1,4-heptanediol, 4-methyl-2,6-heptanediol and 4-methyl-1,6-heptanediol. Metabolism strongly favored the formation of diols and carboxylic acids. The metabolites were identified using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Histopathologic examination of the kidneys revealed minimal hyaline droplet formation (α_2 -globulin nephropathy) in the proximal tubule area.

INTRODUCTION

4-Methylheptane (4-MH), an isomer of octane is a component of gasoline and gasoline based combustion engines [1,2]. In addition to combustion exhaust, general environmental exposure to 4-MH occurs due to leakage of fumes from fuel oil storage tanks and landfill emissions [3,4]. 4-MH has been found as a component of polymer fire retardant mixtures [5] as well as a volatile constituent of cooked red and white meat [6,7]. Similar to other isomers of octane, when injected into animals, 4-MH has been shown to produce hemorrhage, edema and polymorphonuclear

leukocytic reactions such as angitis, abscess formation, thrombosis and fibrosis [8].



4-METHYLHEPTANE

Due to the wide dispersion of 4-MH in the environment, it is extremely likely that many people will be exposed to various concentrations of the chemical. It was, thus, decided to examine how the hydrocarbon is metabolized in animals to determine if potentially hazardous metabolites were formed and to compare the pathway of 4-MH biotransformation with the metabolic pathways of related isomers of octane. Male rats were selected as the subjects of the 4-MH metabolic study. Male rats are frequently used to study the biotransformation of xenobiotic materials because of the similarity of metabolic reactions that exist between man and the rat. Secondly, certain branched chain isomers of octane e.g. 2,2,4-trimethylpentane (2,2,4-TMP) and 2,3,4-trimethylpentane (2,3,4-TMP) have been reported to induce a proximal tubular nephropathy in male rats [9,10]. This nephropathy is characterized by the inability of the rat to breakdown the testosterone controlled protein $\alpha_2\text{u}$ -globulin through the normal lysosomal enzyme processes [11]. 4-MH, similar to other hydrocarbons, is a totally non-polar molecule. Hydrocarbons once in the animal body, are normally eliminated by either exhalation or excretion via the urine. The latter process requires metabolic conversion of the hydrocarbon into a water soluble derivative. It is the production of metabolites with certain functionalities, which, by tying up the $\alpha_2\text{u}$ -globulin and inhibiting its degradation, is generally considered to be responsible for the hydrocarbon-induced nephropathy cited above. A comparison of the ability of 4-MH to 2,2,4- and 2,3,4-TMP to induce the male rat nephropathy would yield interesting information regarding the extent and positioning of structural branching necessary to induce the nephrotoxicity.

MATERIALS

4-Methyl-1-heptanol, 4-methyl-1,4-heptadiene, 4-methyl-1,5-heptadiene, 4-methyl-1-hepten-4-ol acetate, and 4-methyl-1-hepten-3-ol were purchased from Wiley Organics, Coshocton, OH. Di-n-propyl acetic acid was obtained from Lancaster Synthesis, Windham, NH. 4-Methyl-1-heptanol was acquired from Narchem corporation, Chicago, IL. 2-Methyl-4-hydroxy-1-pentanoic acid was synthesized according to the literature procedure [12]. 4-Methyl-1,4-heptanediol was prepared via the mercuric acetate-sodium borohydride reaction of 4-methyl-1-hepten-4-ol acetate [13]. 4-Methyl-1,6-heptanediol was synthesized by the hydroboration of 4-methyl-1,5-heptadiene [14]. 4-Methyl-2,5-heptanediol and 4-methyl-2,6-heptanediol were obtained by the mercuric acetate-sodium borohydride reaction of 4-methyl-1,5-heptadiene [15]. 4-Methyl-1,7-heptanediol was prepared by the lithium aluminum hydride reduction of diethyl 4-methyl-pimelate [16].

METHODS

Twelve Fischer 344 male rats weighing 230 +/- 15 grams were randomly divided into two groups (8 treated, 4 control). Doses (0.8 g/kg) of 4-MH were administered by gavage on an every other day regimen for two weeks. Feed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) and water were provided ad libitum and animals were weighed daily.

Following the 14 day exposure period, the rats were sacrificed by halothane overdose and the kidneys were excised 24 hours following the final dose. Histopathologic examination was performed on paraffin embedded kidney sections stained with hematoxylin and eosin. Tissues from treated rats were compared to controls for characteristic lesions of hydrocarbon-induced nephropathy, including hyaline droplet formation, tubular cysts and papillary calcification. Lesions were graded by pathologists for degree of severity.

During the first 48 hours of the initial dosing period, the rats were placed in metabolism cages and the urine collected. A 5.0 mL aliquot of each urine sample was adjusted to a pH of 4.0 and 0.2 mL glucuronidase/sulfatase (Calbiochem, La Jolla, CA) was added. The sample

was shaken for 16 hours at 37°C, then cooled to room temperature and filtered through a diatomaceous earth column (Chem Elut, Varian International Harbor, CA) using methylene chloride as the eluent.

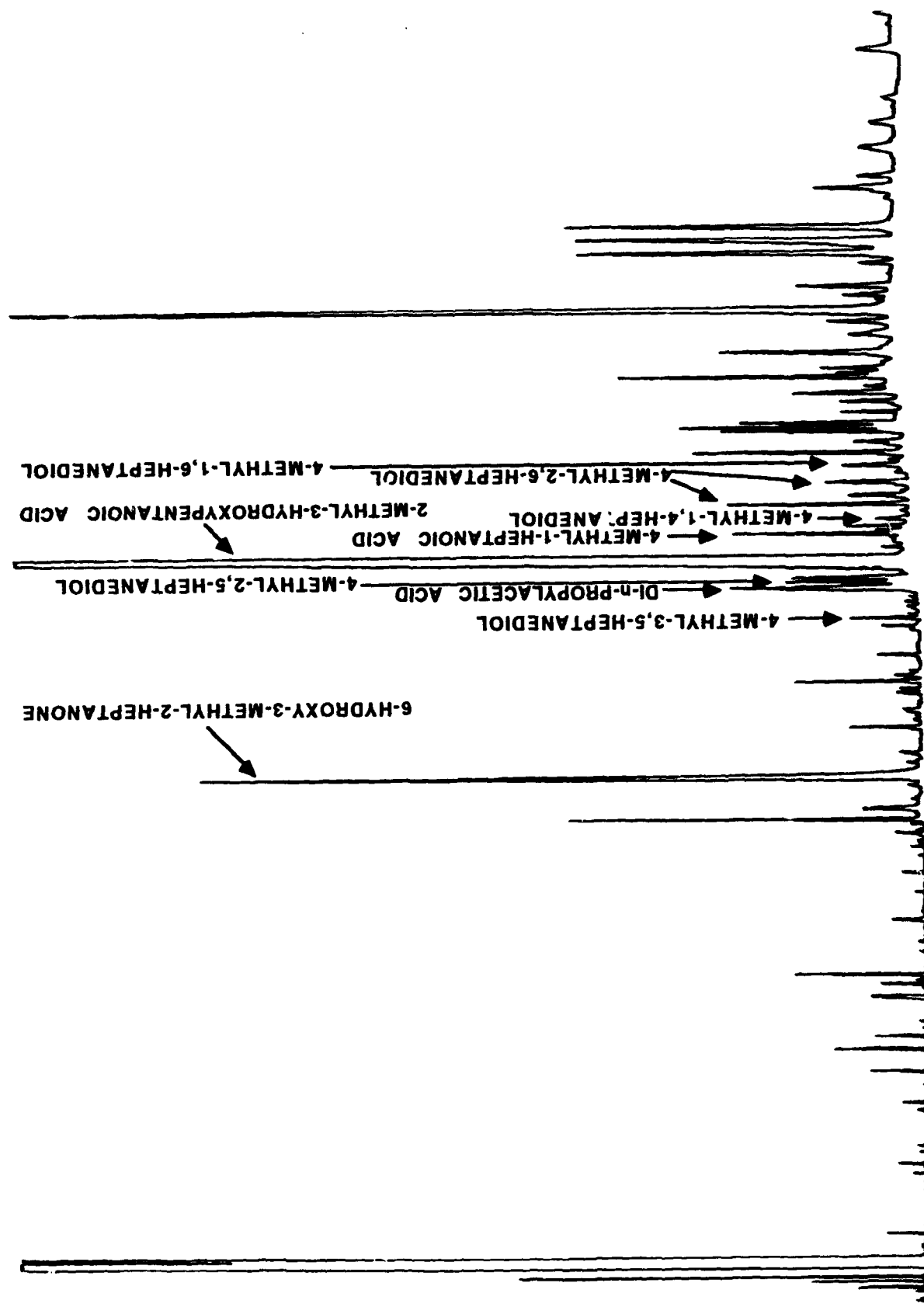
The methylene chloride extracts of the hydrolyzed rat urine were analyzed on a gas-liquid chromatograph (GC) equipped with a flame ionization detector (model 3500, Varian Corp., Walnut Creek CA). A 30 m x 0.32 mm I.D. Carbowax 20M on polyethylene glycol column (Alltech Associates Inc., Deerfield IL) with injection port and detection temperatures of 250°C. The oven temperature was programmed to rise from 100°C to 200°C at a rate of 5°C/min and helium was used as the carrier gas. Additional metabolite identification was accomplished using a Finnegan MAT INCOS 50 -Varian 3400 gas chromatography/mass spectrometer (GC/MS) system (Varian Corp., Walnut Creek, CA). The GC was equipped with an identical column as the gas chromatograph above while the injection port and the oven temperatures were the same as previously reported. Helium was the carrier gas. The MS was a quadrupole instrument operated in the electron impact mode with a voltage of 70eV and an ion source of 200°C.

The difficulty in totally separating all the products (including control metabolites) in the rat urine made quantitation of the metabolites impossible. However, relative areas under the peaks, listed in Table 1, were obtained from the GC program.

RESULTS AND DISCUSSION

After hydrolysis with glucuronidase/sulfatase, the individual urine samples from the rats dosed with 4-MH were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). A representative GC chromatogram is shown in Figure 1. The metabolites names and structures, which correspond to their appearance in the GC chromatogram are presented in Tables 1 and 2, respectively. GC chromatograms of 4-MH dosed rat urines, not treated with glucuronidase/sulfatase, showed no trace of 4-MH metabolites.

FIGURE 4. METABOLITES ISOLATED FROM THE URINES OF MALE FISCHER
344 RATS DOSED WITH 4-METHYLHEPTANE



From Table 2 it was noted that every carbon of the 4-MH molecule underwent metabolic oxidation. An examination of the metabolite structures indicated that the attack at the penultimate carbon position was particularly favored by the biotransformation enzymes. Although diols and ketone structures were found, the metabolism of 4-MH yielded a variety of carboxylic acids. Of note was the isolation of di-n-propyl acetic acid also known as valproic acid. Valproic acid is used as an anti-seizure drug and has been shown to be an inducer of the mixed function oxidase system [17]. The formation of valproic acid can be viewed as the end result of the oxidation of the 4-methyl substituent to the corresponding alcohol followed by oxidation to the aldehyde. No trace of either the precursor alcohol or aldehyde was detected. The oxidation of the 4-methyl substituent was akin to the previously reported metabolic oxidation of the 3-methyl substituent in 3-methylheptane [18].

An unexpected metabolite was 2-methyl-3-hydroxy-1-pentanoic acid. This molecule, which was a major metabolite, revealed the loss of two carbon atoms from the starting 4-MH. Neither 2- or 3- methylheptane underwent metabolism, which resulted in the isolation of compounds where carbon atoms were lost. Previously, n-octane has been shown to lose carbon atoms during biotransformation with the carbons leaving as CO₂ [19]. Similar to n-octane, the loss of the carbon atoms from 4-MH resulted in oxygenated carboxylic acids as the products. This suggests, in order for carbon atoms to be lost via metabolic oxidation, that the hydrocarbon structure must have at least three unsubstituted carbons from either end of the molecule.

Unlike the other metabolites whose structures were confirmed by independent synthesis, the structure of 6-hydroxy-4-methyl-2-heptanone was deduced by its mass spectrum fragmentation pattern and reduction of a metabolic urine sample. Sodium borohydride reduction of an ether solution of the urinary metabolites resulted in a loss in GC peak for 6-hydroxy-4-methyl-2-heptanone and an increase in the GC peak for 4-methyl-2,6-heptanediol, a previously identified metabolite.

Analysis of the kidneys of the rats dosed with 4-MH show only a very slight nephropathy. Histopathologic results indicated that 4-MH graded out on a level of 1 (based on a scale of 0 to 4 where 0 indicated no damage and 4 indicated severe damage). A grade of 1 suggests minimal

damage to the proximal tubule. n-Octane graded out at a level of 0 whereas 2,5-dimethyl-hexane (2,5-DMH), another isomer of octane produced nephrotoxic damage rated at a level of 3. 2,2,4- and 2,3,4-TMP produced severe damage to the male rat proximal tubule which graded out to a level of 4. In none of the rats dosed with 4-MH was there any indication of cast formation. There was no trace of any 4-MH metabolites extracted from the homogenized kidneys of the dosed rats. No hyaline droplet formation was noted in the control animals. Comparing the hydrocarbon induced renal tubular nephrotoxicity of several octane isomers resulted in the following: n-octane < 4-methylheptane < 2-methylheptane < 3-methylheptane < 2,5- dimethylhexane < 2,2,4-trimethylpentane. This suggests that if the hydrocarbon can be metabolized by loss of carbon atoms, the resulting metabolites will be less nephrotoxic. In summary changing the position of the methyl substituent on a simple molecule like heptane changes not only the degree of hydrocarbon induced nephrotoxicity, but also has a bearing on the metabolic pathways by which the hydrocarbons are biotransformed for excretion.

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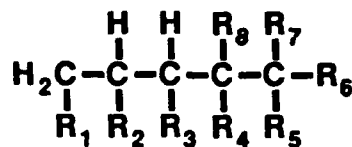
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TABLE 1. METABOLITES ISOLATED FROM THE URINES OF MALE FISCHER 344 RATS
DOSED WITH 4-METHYLHEPTANE

G C PEAK	METABOLITE	RELATIVE ABUNDANCE
1.	6-HYDROXY-4-METHYL-2-HEPTANONE	27.5
2.	4-METHYL-3,5-HEPTANEDIOL	2.3
3.	DI-n-PROPYL ACETIC ACID	6.5
4.	4-METHYL-2,5-HEPTANEDIOL	3.1
5.	2-METHYL-3-HYDROXPENTANOIC ACID	24.6
6.	4-METHYL-1-HEPTANOIC ACID	3.3
7.	4-METHYL-1,4-HEPTANEDIOL	1.0
8.	4-METHYL-2,6-HEPTANEDIOL	4.7
9.	4-METHYL-1,6-HEPTANEDIOL	1.7

TABLE 2 ACYCLIC DERIVATIVES ISOLATED FROM THE URINES OF RATS DOSED WITH 4-METHYLHEPTANE



METABOLITE PEAK NUMBER	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈
1	H	=O	H	H	H	CHCH ₃ OH	H	CH ₃
2	H	O-H	H	H	O-H	C ₂ H ₅	H	CH ₃
3	H	H	H	H	H	C ₂ H ₅	H	CO ₂ H
4	H	O-H	H	H	O-H	C ₂ H ₅	H	CH ₃
5	H	H	O-H	H	=O	O-H	---	CH ₃
6	H	H	H	H	H	CH ₂ OH	H	CH ₃
7	O-H	H	H	O-H	H	C ₂ H ₅	H	CH ₃
8	H	O-H	H	H	H	CHCH ₃ OH	H	CH ₃
9	O-H	H	H	H	H	CHCH ₃ OH	H	CH ₃

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GENERAL DISCUSSION AND CONCLUSIONS

From the metabolism of the various isomers of octane, including the compounds examined in this project, the following conclusions can be developed:

- 1). straight chain hydrocarbons, when metabolized by rats seem to be susceptible to loss of carbon atoms.
- 2) hydrocarbons possessing a methyl substituent at either one or two carbons from the end of the molecule do not suffer loss of carbons during metabolism.
- 3) hydrocarbons with a methyl group that is three carbons from the terminus of the molecule appear to act like straight chain molecules in that metabolism yields metabolites containing fewer carbon atoms than the starting hydrocarbon.
- 4) metabolite structures of hydrocarbons contain the following types of functional groups: alcohols, diols, hydroxy acids, keto acids and carboxylic acids.
- 5) the order of increasing α_2 -globulin nephrotoxicity for the compounds examined in this study along with previously examined hydrocarbons is:
n-octane < 4-methylheptane $\leq$ 2-methylheptane < 3-methylheptane < 2,5-dimethylhexane < 2,2,4-trimethylpentane < 2,3,4-trimethylpentane.
- 6) a recent article on the characteristics of chemical binding of metabolites of hydrocarbons to α_2 -globulin indicates that two structural features should be present (a) a lipophilic region and (b) an electronegative atom to accept a hydrogen atom and form a hydrogen bond between the metabolite and the α_2 -globulin [51]. This information along with the finding that chemically bound α_2 -globulin is more resistant to hydrolysis [52] would seem to indicate that the structure of the metabolites found for the hydrocarbons investigated in this study, although similar in functional groups, possess different abilities to bind to α_2 -globulin and thus permit the differing degrees of nephrotoxicity found.

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