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THE CYTOTOXIC EFFECTS OF TRIMETHYLPENTANE ON RAT RENAL TISSUE

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Abstract

The primary objective of the investigation was to determine the acute cytopathologic effects of 2,3,4 - trimethylpentane, a major constituent of gasoline, on renal tissue of the mature male rat. Three groups of 9 Fischer-344 rats each were administered trimethylpentane by gavage twice weekly for 7, 14, or 28 days at a concentration of 1.5 ml/kg body weight. The tissues were fixed by perfusion, and subsequently processed for scanning and transmission electron microscopy. Although the manifestations of hydrocarbon toxicity were evident in all experimental tissues examined, the extent and magnitude of cellular lesions increased as the exposure period progressed. The only structural change detected within the glomerular complex was a significant increase in the number of microvilli associated with various branches of the podocytes. Cells of the proximal convoluted tubule were characterized by the presence of membrane - bound, PAS positive hyaline droplets. At focal points along segments P_1 and P_2 of the proximal tubule intact epithelial cells dissociated from the basal lamina, underwent necrosis, and subsequently collected along the length of the tubular lumen. The cellular debris concentrated at the corticomedullary junction. Tubules at the site were dilated focally and the epithelial lining was attenuated. Results of the study indicate that the manifestations of trimethylpentane toxicity among renal cells are associated with the proliferation of hyaline droplets.

KEY WORDS: Hydrocarbon toxicity, kidney, scanning electron microscopy, gasoline, glomerulus, proximal convoluted tubule, cytotoxicity.

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Introduction

Interest in hydrocarbon cytotoxicity has increased substantially with the revelation that male rats chronically exposed to gasoline vapors develop nephropathy and demonstrate a significant increase in the incidence of renal carcinomas (MacFarland, 1984). There are also a multiplicity of petroleum-based and synthetic hydrocarbon fuels which are selectively nephrotoxic for male rats (Bruner and Pitts, 1983). The manifestations of hydrocarbon cytotoxicity with regard to renal tissue include hyaline deposition among epithelial cells of the proximal convoluted tubule, proximal tubular cell degeneration, intratubular casts, and carcinomas for chronic studies of at least a duration of 1 year (Olson et al., 1985). Unresolved is the precise mechanism which governs the transformation of renal epithelial cells into neoplasms subsequent to chronic exposures to selective hydrocarbons. However, the pathogenesis of early renal changes associated with hydrocarbon toxicity should be investigated to determine possible correlations regarding cellular lesions resulting from initial and chronic exposures of the exogenous compounds.

This study was designed to elucidate by means of scanning and transmission electron microscopy the various ultrastructural manifestations of 2,3,4-trimethylpentane on renal tissue of the sexually mature male rat. Trimethylpentane (isooctane) is a principal constituent of gasoline and represents the standard reference fuel for assigning "octane rating". Olson et al. (1985) have reported on the urinary metabolites of trimethylpentane in male rats. However, a comprehensive ultrastructural investigation regarding the cytotoxic consequences of acute exposure of trimethylpentane to the male rat has yet to be conducted. This paper represents such a study.

Materials and Methods

The hydrocarbon 2,3,4-trimethylpentane was obtained from Aldrich Chemical Company. Three groups of 9 Fischer-344 rats each were dosed by gavage twice weekly for 7, 14 or 28 days,

respectively, at a concentration of 1.5 ml/kg body weight. Fifteen control animals were administered by gavage a comparable concentration of distilled water. Experimental and control animals were sacrificed at 7, 14 or 28 days subsequent to the initial exposure. All tissues selected for analysis were fixed by perfusion via the dorsal aorta at a hydrostatic pressure equal to 150 cm water.

The perfusate was 1% glutaraldehyde in 0.135M Sorensen's phosphate buffer (pH 7.2). After a perfusion period of 3 minutes, the kidneys were excised and minced. Small segments of tissue were fixed for 1h by immersion in a fresh 0.135M Sorensen's phosphate buffered solution of 4% glutaraldehyde. The tissues were rinsed several times in 0.1M sodium cacodylate, and postfixed for 1h at 25°C in a 0.1M sodium cacodylate buffered solution of 1% osmium tetroxide. Subsequent to several rinses in fresh buffer, the tissues were dehydrated in a graded series of ethanol. The specimens were separated into 2 groups and further processed for scanning and transmission electron microscopy, respectively. Samples selected for TEM were passed through 3 changes of propylene oxide and embedded in Epon 812. Thin-sections were cut by a Sorvall MT2 ultramicrotome, and stained with lead citrate and uranyl acetate. The sections were examined in a JEOL 100B transmission electron microscope at 60kV.

Tissues chosen for analysis by SEM were placed into small cylinders of parafilm which contained absolute ethanol. Both ends of each cylinder were crimped shut. The cylinder and its internal contents of ethanol and tissue were frozen in liquid nitrogen. The cylinder was placed on a flat metal block which had been precooled in liquid nitrogen. A precooled, single-edge razor blade was used to make a fracture through the cylinder and tissue. The fractured fragments were placed in absolute ethanol, and critical point dried using liquid CO_2 as described by Anderson (1951). The dried tissues were attached onto metallic stubs with silver conducting paint, and coated with a thin layer of gold. Specimens were observed in an ETEC Autoscan scanning electron microscope at 20kV.

Subsequent to perfusion, sections of kidney selected for a periodic acid-Schiff reaction were fixed for 24 hours in a neutral 10% formalin solution buffered with sodium acetate. Further processing of the tissues and the specific histochemical procedures were conducted according to the method described by Lillie and Fullmer (1976).

Results

The various manifestations of hydrocarbon toxicity were evident in all experimental tissues examined. However, the extent and magnitude of cellular lesions as monitored by general observations of experimental tissue increased gradually as the exposure period progressed. All exposures were sublethal.

The overall structural integrity of the glomerular complex was maintained throughout the duration of the study. The sole ultrastructural abnormality noted within the glomerular region was detected by scanning electron microscopy. As compared to control tissue (Fig. 1) there was a significant increase in the number of microvilli (Table 1) associated with the primary, secondary and tertiary branches of podocytes (Fig. 2). There was no discernible disruption of the endothelial lining or swelling of the basal lamina (Fig. 3). The pedicels and their associated filtration slits appeared normal.

Table 1

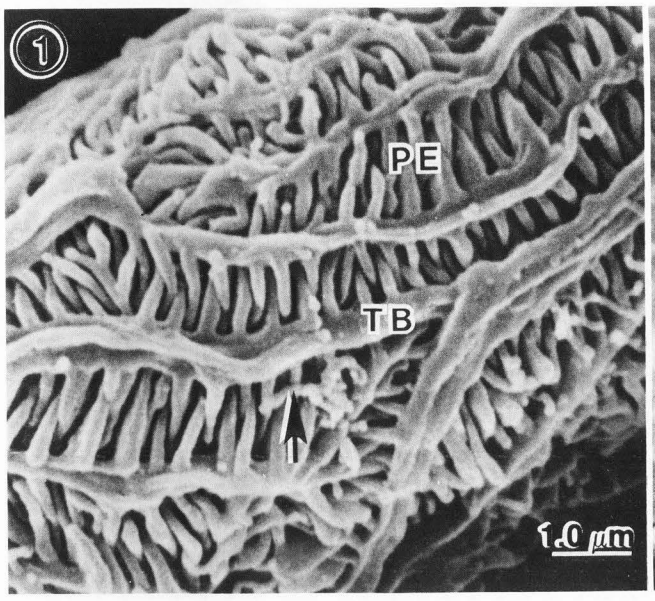
The number of glomerular microvilli per unit area. Values were obtained from 6 rats for each of the specific time points and represented an analysis of 10 random sites per organ.

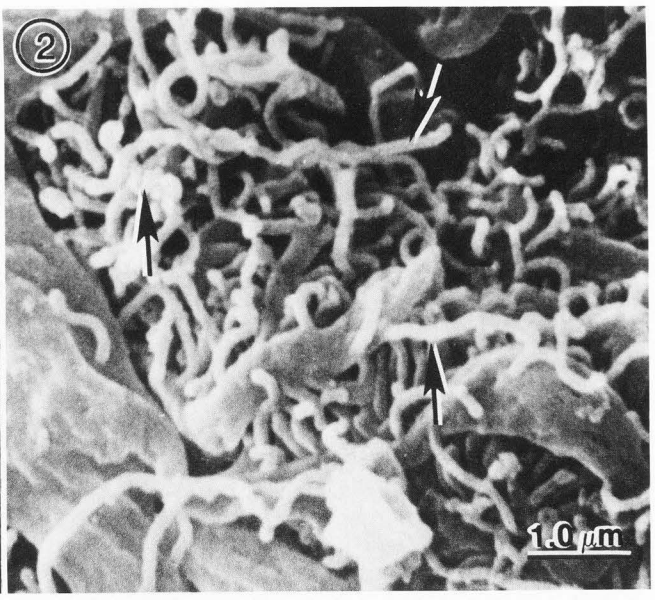
Exposure in days	Number of microvilli
Control	6.7±2.7*
7	10.5±3.2*
14	16.5±1.9*+
28	24.3±5.1*+

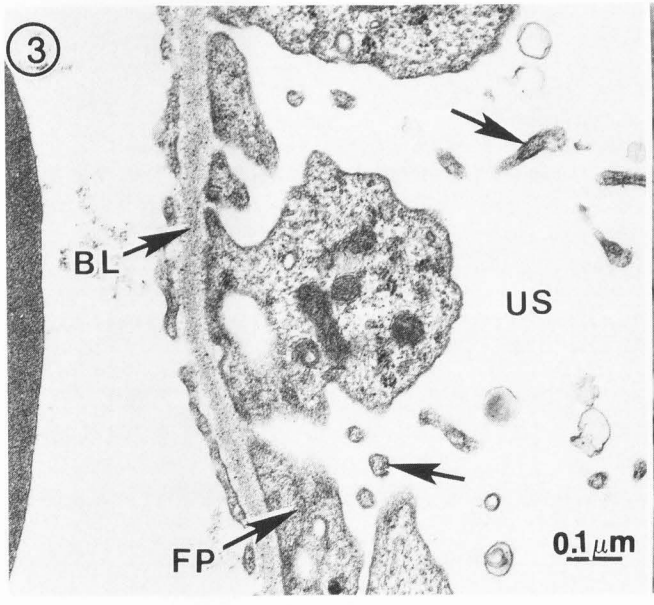
* Mean and standard error
\+ Significant at the 0.05 confidence level

At focal sites along the length of segments P_1 and P_2 of the proximal convoluted tubule, epithelial cells displayed evidence of structural alteration. The most prominent feature of the affected cells was a marked increase in the number and size of pleomorphic hyaline droplets. The primary sites of proliferation consisted of cells comprising segment P_2 of the proximal tubule, while the structures were evident to a lesser degree in segment P_1. The hyaline droplets stained positive for the periodic acid-Schiff reaction (Fig. 4). The membrane-bound droplets were present as spherical bodies and irregularly shaped structures which possessed angular vertices (Figs. 5 and 6). There appeared to be a sequential pattern to the formation of the specific shapes assumed by the droplets. Initially the spherical bodies were evident in affected cells, however, as the manifestations of toxicity intensified, the relatively large angular configurations appeared to be more prevalent. The hyaline droplets characterized by the angular shape displayed a crystalline core which demonstrated a periodicity of 3.57 nm (Fig. 7). Such a precise pattern was not evident in the spherical droplets.

The effects of trimethylpentane toxicity among mitochondria of cells comprising the proximal tubule were evident in several structural anomalies. Many of the cells which contained hyaline droplets possessed mitochondria







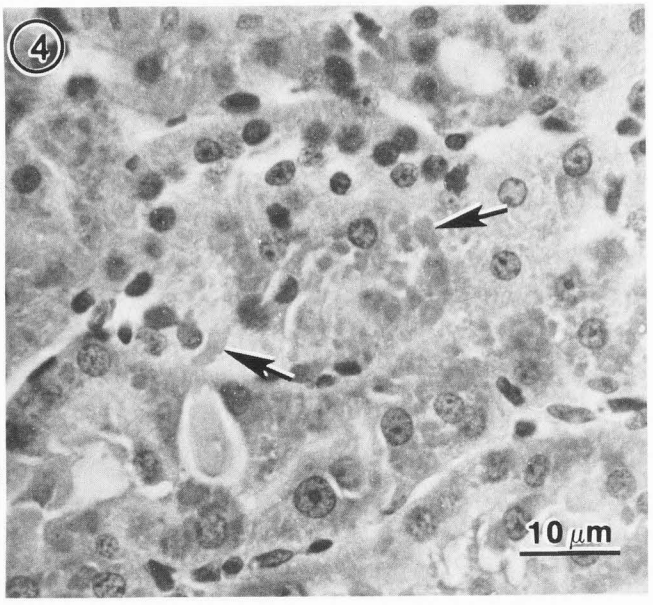


Figure 1. Surface of glomerular complex with orderly arranged pedicels (PE) and tertiary branches (TB) of podocytes. Note sparsity of microvilli (arrow) - Control tissue.

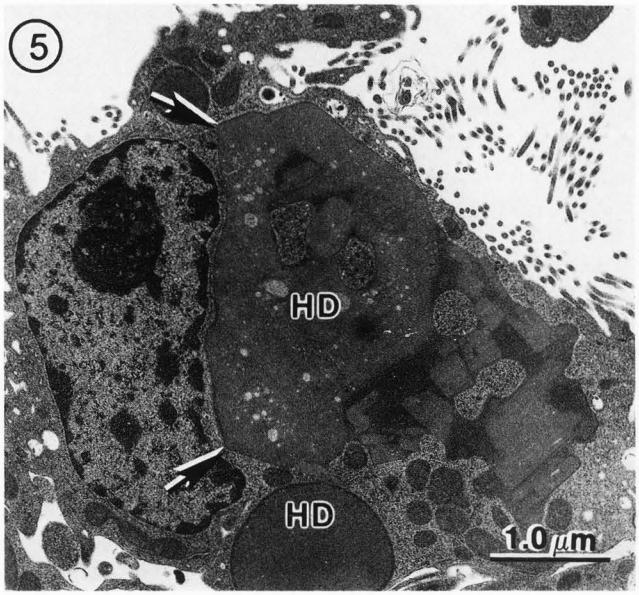
Figure 2. Proliferation of microvilli (arrows) along the length of glomerular complex. Experimental tissue - 28 days of exposure.

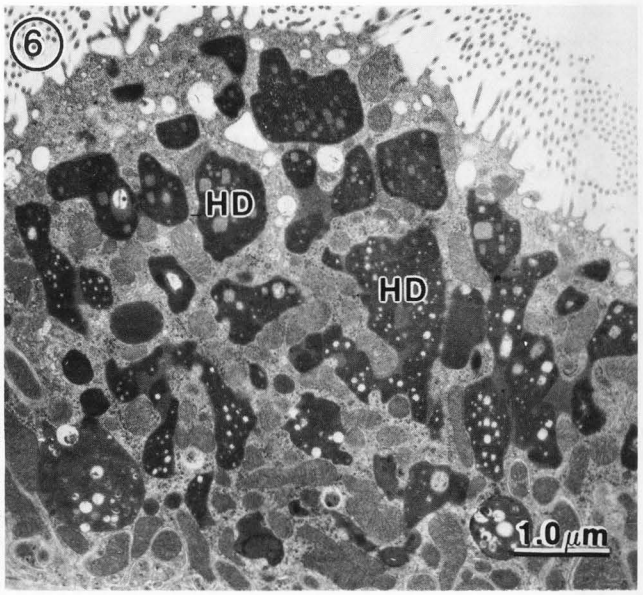
Figure 3. Multiple microvilli (arrows) extend into urinary space (US). Basal lamina (BL) and foot processes (FP) of pedicels appear normal. Experimental tissue - 14 days of exposure.

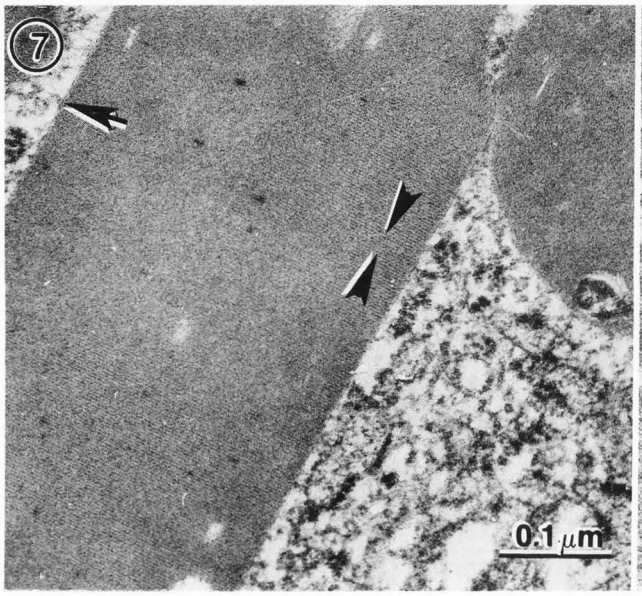
Figure 4. Brightfield photomicrograph reveals the presence of PAS positive hyaline droplets (arrows). Proximal tubule associated with the pleomorphic droplets appears disrupted. Experimental tissue - 14 days of exposure.

that appeared either swollen (Figs. 8 and 9) or in a state of degradation as indicated by the presence of myelin figures. Also evident were mitochondria which contained an electron-dense matrix.

The apical canaliculi displayed no evidence of structural aberrations. Electron-dense coated vesicles were observed at the base of apical microvilli in all tissues examined. An analysis of segments P_1 and P_2 of the proximal tubules revealed distinct regions along the inner lumen where a loss of microvilli was evident (Figs. 9







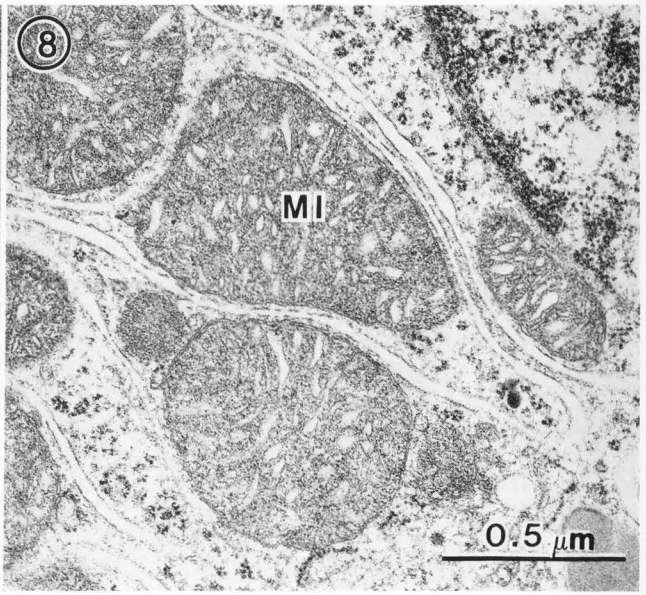


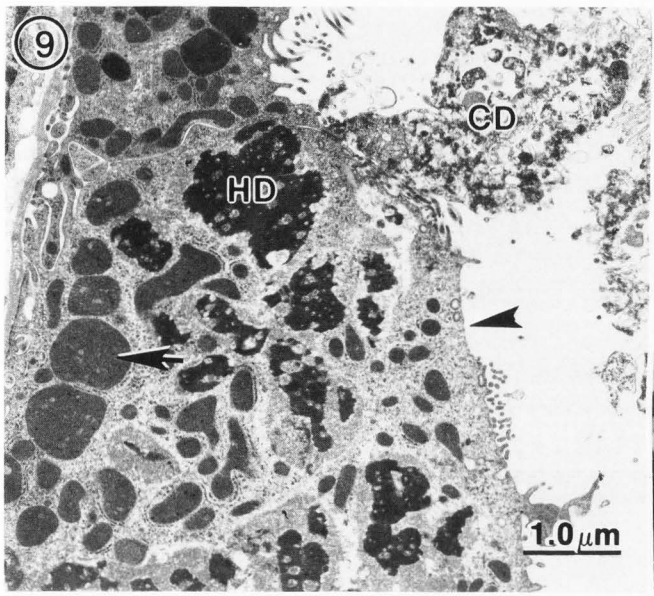
Figure 5. Proximal tubular cell contains several hyaline droplets (HD), one of which is characterized by angular vertices (arrows). Experimental tissue - 7 days of exposure.

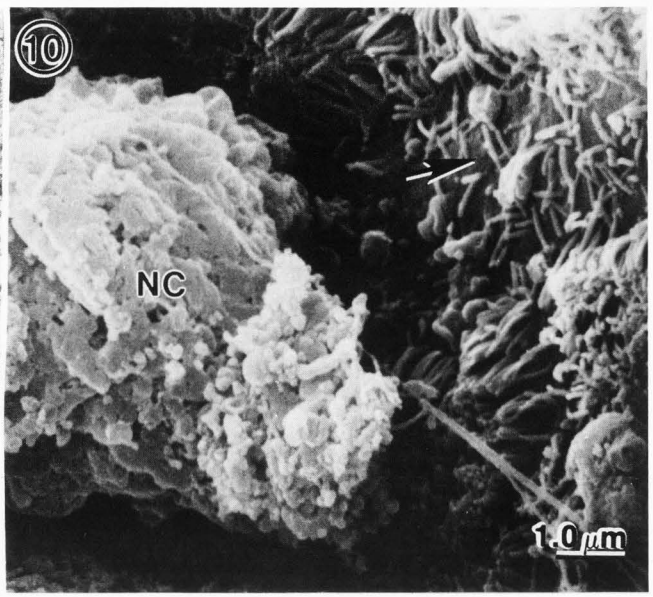
Figure 6. Proximal tubular cell displays an abundance of hyaline droplets (HD) which are irregular in size and shape. Experimental tissue - 28 days of exposure.

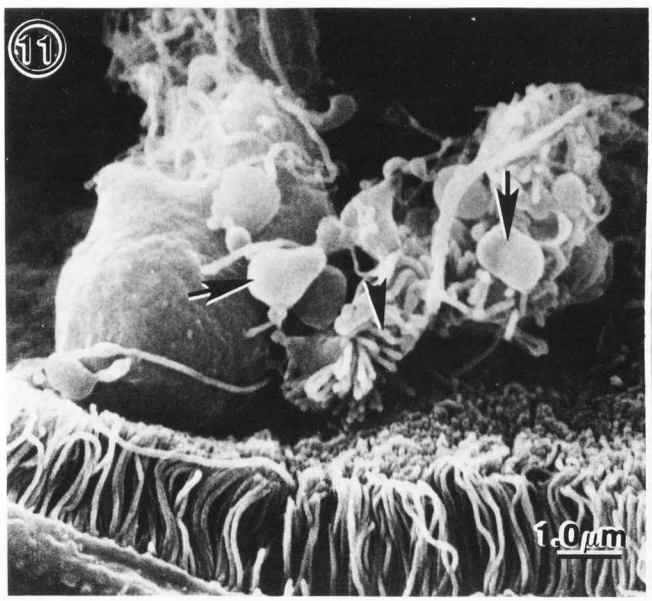
Figure 7. Hyaline droplet is membrane-bound (arrow) and demonstrates an internal periodicity (arrowheads). Experimental tissue - 14 days of exposure.

Figure 8. Several swollen mitochondria (MI) with slightly dilated cristae are evident in a sensitive cell of the proximal tubule. Experimental tissue - 7 days of exposure.

and 10). In several instances, the denudation of microvilli was noted among individual epithelial cells which dissociated from the basal lamina and collected in the lumen (Figs. 9-11). There was little evidence of cellular fragmentation prior to the dissociation. However, once within the lumen, cells appeared to undergo autolysis (Figs. 9 and 11). Subsequently, the cellular debris tended to concentrate in the corticomedullary junction. The tubules were dilated focally and the epithelial lining was markedly attenuated (Fig. 12).







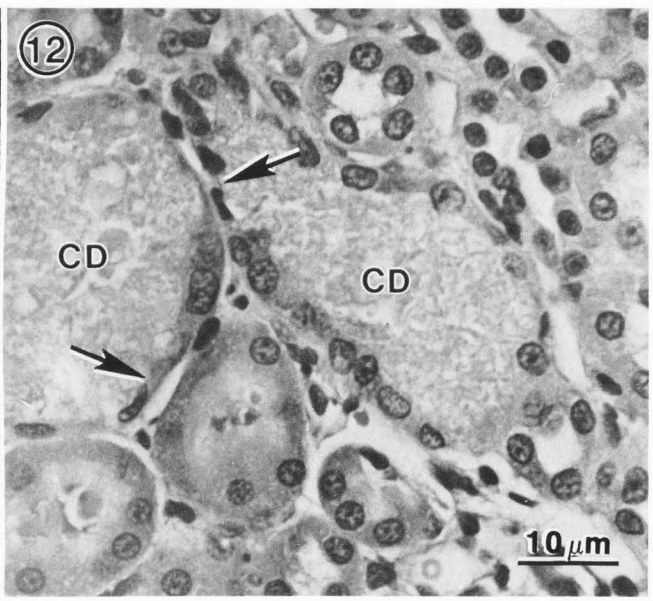


Figure 9. Proximal tubular cell is characterized by swollen mitochondria (arrow), hyaline droplets (HD) and reduced concentration of microvilli (arrowhead). Note cellular debris (CD) in lumen. Experimental tissue - 14 days of exposure.

Figure 11. Hyaline droplets (arrows) and microvilli (arrowhead) have been released from fragmented epithelial cell into lumen of proximal tubule. Experimental tissue - 14 days of exposure.

Figure 10. Necrotic cell (NC) is positioned in lumen of proximal tubule. Adjacent region has a reduced content of microvilli (arrow). Experimental tissue - 7 days of exposure.

Figure 12. Substantial accumulation of cellular debris (CD) in the general region of the corticomedullary junction results in dilation of the tubules and attenuation of the epithelial lining (arrows). Experimental tissue - 28 days of exposure.

Discussion

The determination that exposure of gasoline vapors to male rats results in nephrotoxicity and carcinogenicity (MacFarland, 1984) suggests a potential risk to the general population exists. However, an interesting facet of this issue and one which may impact on risk assessments concerns the inability of hydrocarbons associated with gasoline to induce cellular lesions or neoplastic changes in other organs and female rats. There is an apparent relationship between

trimethylpentane toxicity and the proliferation of hyaline droplets among cells of the proximal tubule. A variety of petroleum-based and synthetic hydrocarbons have proven to be initiators of nephrotoxicity and, more specifically, inducers of hyaline droplet formation (Bruner, 1984; Halder et al., 1984). Investigators have speculated that accentuated hyaline droplet production may be dependent upon a sex related, low molecular weight protein, alpha 2μ globulin (MW 26,400) (Bruner, 1984; Irwin et., 1971). The protein is synthesized by the liver of male rats at puberty under the influence of testosterone. The globulin is readily filtered through glomeruli of the kidney, and represents the primary urinary protein of male rats (Roy, 1973; Roy and Neuhaus, 1966; 1976). There is no indication that the protein is synthesized by female rats.

Hyaline droplets represent structures which usually result from absorption of proteins from the glomerular filtrate. Subsequently, the proteins concentrate in large apical vesicles which are transformed into lysosomes (Kretchmer and Bernstein, 1974). Occasionally, the hyaline droplets represent an expression of disturbed cellular metabolism. For example, droplets develop in cells of collecting tubules during potassium deficiency, and are a characteristic feature of malignant nephrosclerosis (France, et al., 1974; Churg et al., 1980).

Bruner (1984) has postulated that among male rats exposed to selective hydrocarbons, the alpha 2μ globulin represents the major constituent of hyaline droplets, and the biochemical and cytological consequences of its excessive accumulation in proximal tubular cells relate to the manifestations of cytotoxicity. The accentuated concentration of droplets may reflect an inability to degrade enzymatically what would normally be an innocuous protein, once uptake has occurred. An investigation should be conducted to determine the extent, if any, to which the activity of lysosomal enzymes are affected by trimethylpentane.

The positive periodic acid-Schiff reaction demonstrated by hyaline droplets present in epithelial cells of kidney exposed to trimethylpentane indicates the presence of a carbohydrate moiety. Results of the PAS reaction tend to support the supposition that the structures possess hyaline since previous studies have verified the presence of carbohydrates in hyaline droplets (Churg et al., 1980).

Ultrastructural observations indicate that as the hyaline droplet content of tubular cells increases during progressive exposure, there is a concomitant change in the three-dimensional configuration of the structures from spherical to angular bodies. The transformation in shape may be correlated with a chemical or physical alteration of the internal contents, since analysis of the spherical bodies reveal no discernible substructure, whereas the angular droplets are characterized by a periodicity. Such a crystalline formation indicates that a proteinaceous complex, conceivably alpha 2μ globulin, concentrates in the hyaline droplets as a result of exposure to trimethylpentane. The apparent relationship between elevated levels of hyaline droplets and the dissociation of epithelial cells from the basal lamina represents a phenomenon which currently is unresolved. There appears to be no degradation of the basal lamina prior to the dissociation, and the plasma membrane maintains its integrity until the cell settles in the lumen.

Cells aligning the compromised segments of proximal tubules display no evidence of the classical manifestations of regeneration such as an abundance of free polysomes, a paucity of other organelles and a large lobed nucleus with diffuse chromatin. However, during a period of recovery cellular regeneration would seem possible since the basal lamina remains intact structurally, and significant numbers of cells in the affected segments appear to be unaltered by trimethylpentane. Renal tissue of rats exposed to hydrocarbons has demonstrated an ability to recover physiologically from toxic insults induced by the compounds. D'Addario et al. (1985) report that rats continuously exposed to jet fuel vapor for 90 days experience a significant reduction in the urine concentrating ability of the kidneys. All biochemical indicators return to control levels following a 9 week recovery period.

Whereas the consistent proliferation of hyaline droplets indicates an indirect effect of trimethylpentane toxicity, the transformations detected among mitochondria regarding swelling and degradation intimate direct manifestations of hydrocarbon exposure. Comparable toxic effects on mitochondria have been reported for hepatocytes of fat-head minnows exposed to aromatic hydrocarbons (Norton et al., 1985). The extent of mitochondrial dysfunction among tubular cells has not been determined. However, the organelles display structural changes which indicate both reversible and irreversible damage.

Epithelial cells of the proximal tubule which contain prominent hyaline droplets undergo a marked reduction in the number of apical microvilli. Such an effect is not unexpected since microvilli are highly sensitive to injury and frequently respond by becoming ballooned or reduced in number (Churg et al., 1980). However an interesting consequence of trimethylpentane exposure on the glomerular complex concerns the significant increase in number of microvilli associated with podocytes. The authors are not cognizant of other conditions which may induce such a selective effect. Hydrocarbons appear to alter cilia in just the opposite manner. Epithelial cells of the nasal mucosa of fat-head minnows exposed to aromatic hydrocarbons are characterized by a loss of cilia (Norton et al., 1985).

In summary, this investigation represents an initial attempt to analyze by scanning and transmission electron microscopy the ultrastructural effects of an acute exposure of trimethylpentane to renal tissue of the sexually mature rat. Several pertinent manifestations of toxicity are evident, including the dissociation and subsequent necrosis of epithelial cells from the basal lamina of specific segments along the proximal convoluted tubules. The cytotoxicity of

affected epithelial cells appears to be associated with the proliferation of PAS positive hyaline droplets which consist of crystalline material as demonstrated by an internal periodicity. Future investigations should be designed to determine the biochemical nature of the droplets, and analyze the cause and effect relationship, if any, between the degradation of proximal tubular cells during acute exposure and the carcinogenicity associated with chronic exposure.

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Discussion with Reviewers

A.P. Evan: What characterisics were used to specify the droplets as possessing hyaline?
Authors: Hyaline droplets have been characterized as membrane-bound structures which display a PAS positive reaction (Churg et al., 1980). The PAS positive reaction demonstrated by droplets present in cells sensitive to trimethylpentane was the primary criterion employed to indicate such structures consisted of hyaline.

R. Bulger: What segments (P_1,P_2,P_3) of the proximal tubule have increased hyaline droplets and how does that relate to the segments of the proximal tubule undergoing all injury and necrosis?
Authors: The various manifestations of toxicity, including the proliferation of hyaline droplets and the dissociation of epithelial cells containing droplets from the basal lamina were especially prevalent in segment P_2, while they were evident to a lesser degree in segment P_1.

D.B. Jones: Is there any disturbance of renal function in the rats exposed in this manner to trimethylpentane?
Authors: The various parameters of renal function were not monitored during the investigation. However, D'Addario et al. (1985) have determined that rats exposed to the hydrocarbons comprising jet aviation fuel experience a significant reduction in the urine concentrating ability of the kidneys. All biochemical indicators return to normal levels following a 9 week recovery period.

A.P. Evan: How did the authors determine that the extent and magnitude of the lesion increased with time?
Authors: A morphometric analysis was not conducted during the investigation, therefore, all determinations were based on observations and subjective evaluations of the compromised tubules. As time progressed there appeared to be a greater number of cells which demonstrated manifestations of toxicity.

A.P. Evan: Were the changes dose related?
Authors: Since a constant dose of 1.5 ml/kg body weight was maintained during the period of exposure, the study was not designed to determine dose related responses. The variable in our experimental design was duration of exposure. However, the effects of trimethylpentane on renal tissue of the rat does appear to be dose related (personal communication).

A.P. Evan: How did the authors determine that the droplets increased in number and size?
Authors: A morphometric analysis of the hyaline droplets was not conducted, however, a comparison of cells in similar segments regarding relative size and approximate number of droplets indicated that increases in the 2 parameters occurred as the period of exposure progressed.

A.P. Evan: Were there changes noted along the rest of the nephron?
Authors: The only detectable change occurred in the general region of the corticomedullary junction where the tubules were markedly dilated, apparently as a result of the accumulation of an extensive amount of cellular debris in the lumen (Fig. 12). In addition, many of the epithelial cells lining the dilated tubules were attenuated.

D.B. Jones: Do the lesions go away rapidly after cessation of treatment?
Authors: This study was designed to analyze renal tissue only during the period of exposure. The question concerns a subject which certainly should be addressed in a future investigation.