EFFECTS OF INHALED CHLOROTRIFLUOROETHYLENE
AND HEXAFLUOROPROPENE ON THE RAT KIDNEY

by

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May 1 1980
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To my parents,
for their patience, understanding, and support
throughout my academic career

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ABSTRACT

Male Fischer-344 rats exhibited dose related proximal renal tubular necrosis, diuresis, decreases in the rate of body weight gain, and increases in urinary fluoride (F^-), urinary lactic dehydrogenase (LDH) activity, urinary alkaline phosphatase (ALP) activity, urinary glucose, serum creatinine, and BUN within 2 days following single 4.0 hour inhalation exposures to concentrations of chlorotrifluoroethylene (CTFE) ranging from 100 ppm to 540 ppm, or concentrations of hexafluoropropene (HFP) ranging from 380 ppm to 1200 ppm. The toxicities of CTFE and HFP were similar, with the notable differences being 1) lower threshold concentration for toxicity of CTFE, 2) more prolonged effects from HFP, and 3) necrosis of pars recta and pars convoluta portions of the proximal tubule in HFP exposed rats, but necrosis of the pars recta only in those exposed to CTFE. Near threshold concentrations for CTFE and HFP (about 100 ppm and 380 ppm, respectively) diuresis was the most sensitive index of toxicity manifesting 50% increases in water intake and 25% decreases in urine osmolality. Increases in urinary LDH activity corresponded to proximal tubular necrosis, with greater than 100 fold increases at the highest concentrations of CTFE or HFP. At the threshold concentration for CTFE to induce renal dysfunction (mild diuresis), there was no significant increase in urinary LDH and no apparent necrosis. The threshold concentration for HFP was not found as all concentrations studied produced necrosis. The necrosis was apparent with 24 hours post-exposure, with tubular cell regeneration apparent within 4 days.

INTRODUCTION

As early as 1925 it was learned that fluoroalkenes such as chlorotrifluoroethylene (CTFE) could be polymerized by free-radical catalysis (1). With rapid commercial utilization of polymers, naturally these fluoroalkenes came into common use as industrial chemicals. Upon synthesis by the manufacturer, they were cursorily evaluated with respect to their general toxicity, and subsequently handled with the caution deemed necessary at the time. Accidental exposures in industry, though not common place, brought the toxic potential of these and other industrial chemicals to our attention, causing an increased interest in more intense evaluation of the long term effects as well as more subtle acute effects of these fluorocarbons (2,3). Studies have now shown CTFE and hexafluoropropene (HFP) to produce renal dysfunction in rats, following inhalation exposure (2,4,5).

HFP has been implicated as a pyrolysis product of the polymer polytetrafluoroethylene (PTFE) (6). Waritz and Kwon (6) reported that the major pyrolysis products of PTFE at 300°C were tetrafluoroethylene, octafluorocyclobutene, perfluoroisobutylene, and HFP. Of these pyrolysis products, perfluoroisobutylene and HFP have proved the most toxic.

Exposure to these toxins in the past has often been to workers who handle PTFE contaminated materials prior to smoking a cigarette. The PTFE is transferred from the fingers to the cigarette where it subsequently undergoes pyrolysis when the cigarette is smoked, giving the smoker an inhalation dose of these toxins in addition to the

particulates and toxins normally received from inhalation of cigarette smoke. The symptoms resulting from these exposures include tightness of chest, shortness of breath, cough, chills, and fever. The syndrome of these symptoms has been referred to as "polymer fume fever". In the past "polymer fume fever" has been considered relatively benign. However, in a case study, Brubaker (7) questioned the harmlessness of "polymer fume fever", and suggested the possibility of serious and permanent damage resulting from these exposures.

Chemistry of Fluoroalkenes

CTFE and HFP are two highly volatile alkenes that are completely substituted with halogens. The structures and physical properties of these compounds are as follows:

<u>CTFE</u>	<u>HFP</u>
$\begin{array}{cc} \text{F} & \text{F} \\ & \text{C}=\text{C} \\ \text{F} & \text{Cl} \end{array}$	$\begin{array}{cc} \text{F} & \text{F} \\ & \text{F}-\text{C}-\text{C}=\text{C} \\ & \text{F} & \text{F} & \text{F} \end{array}$
M.W. = 116.5 g/mole	M.W. = 150.0 g/mole
B.P. = -26.2° C @ 1 atm	B.P. = -30.5° C @ 1 atm

Since fluorine is the most electronegative element, the area of the double bond in fluoroalkenes tends to be electron deficient rather than electron rich. The result of this characteristic is that fluoroalkenes are now excellent candidates for nucleophilic attack by bases or other nucleophiles, e.g. OH^- , NH_2 , or SH . The susceptibility of the fluoroalkene to nucleophilic attack increases with the stability of the intermediate carbanion, i.e. $3^\circ > 2^\circ > 1^\circ$ (8). Furthermore, Cook and

Pierce (8) have correlated the toxicity of a halogenated alkene to be directly proportional to the reactivity of that alkene to nucleophiles.

Inhalation Toxicity

The precise *modus operandi* of fluoroalkene inhalation toxicity has yet to be elucidated. Attempts to correlate fluoroalkene toxicity to structure have been only marginally successful (8). For example, trans-2-3-dichlorohexafluorobutene-2 is 3 times more toxic than the cis isomer (9).

Vinyl fluoride ($\text{CH}_2=\text{CHF}$) and vinylidene fluoride ($\text{CH}_2=\text{CF}_2$) are two fluoroalkenes of relatively low toxicity. Rats exposed to 80% vinyl fluoride or vinylidene fluoride for 12.5 and 19.0 hours, respectively, did not die (10). Repeated exposures of male and female rats to 100,000 ppm vinyl fluoride 7 hours/day, 5 days/week, for 30 exposures resulted in no fatalities; the rats gained weight normally, and histology revealed no tissue morphological changes (2).

Fluoroalkenes of slight to moderate toxicity are tetrafluoroethylene, HFP, and CTFE. Animal studies have indicated that these compounds are irritating to the respiratory tract and lungs in lethal concentrations, and histology following exposures has revealed kidney damage (4,5,11).

Fluoroalkenes of high toxicity are cis- and trans-2-3-dichlorohexafluorobutene-2 ($\text{CF}_3\text{CCl}=\text{CClCF}_3$) and perfluoroisobutylene [$(\text{CF}_3)_2\text{C}=\text{CF}_2$]. The relative toxicities of the above mentioned compounds are listed in Table 1. Clayton (2) reported that rats which died from acute inhalation exposure to perfluoroisobutylene exhibited pulmonary

Table 1. Lethal concentrations of several fluoroalkenes.

Fluoroalkene	LC ₅₀ , ppm* (4h exposure)	ALC, ppm**
CH ₂ =CHF		800,000
CH ₂ =CF ₂		128,000
CF ₂ =CF ₂	40,000	
CF ₃ CF=CF ₂	3,000	
CF ₂ =CFCl	1,000	
CF ₃ CCl=CClCF ₃		
trans	61 (1h)	
cis	179 (1h)	
(CF ₃) ₂ C=CF ₂	0.76	

* LC₅₀, lethal concentration for 50% of the animals.

** ALC, approximate lethal concentration, 4h exposure.

edema post mortem, but no discernible effects on other organs.

Chenoweth, as reported by Clayton (2), found that rats subjected to acute exposures to dichlorohexafluorobutene died of severe injury to lungs, liver, and kidney.

CTFE, which is used today in the coated resin ADOGEN 464, was found to be toxic to the rat kidney as early as 1956 (2). In a separate study, Radford (reported by Clayton) exposed groups of male rats to 125, 240, 340, or 460 ppm CTFE for 4 hours. Half of the rats exposed to 460 ppm died. The remaining animals exhibited a slight

decline in body weight and food intake, and dose related diuresis with three fold increases in urine output appearing within 24 hours post-exposure and lasting several days. From Radford's study, the threshold for renal dysfunction appeared to be about 100 ppm CTFE. In the study performed by Hood (reported by Clayton), rats were exposed to CTFE for 4 hours at concentrations of 800, 900, 1000, or 1200 ppm. The LC_{50} was calculated to be 1,000 ppm for a 4 hour inhalation exposure, with death occurring 1-11 days post-exposure. Pathologic examination on these rats revealed pulmonary edema and what was described as degeneration of the renal tubules.

In 1974, Dilley, Carter and Harris (4) found that rats exposed to sublethal concentrations of HFP exhibited periods of slightly elevated fluoride ion excretion and diuresis that persisted up to two weeks with a 50% increase in urine output. Danishevskii and Kochanov (5) found that rats exposed to HFP exhibited glucosuria, diuresis, and proximal tubular damage in the kidney. Pathologic changes noted by Dilley in HFP exposed rats were marked necrosis and dilatation of the proximal tubules.

Methods of Detecting Renal Injury

Since the primary interest in the present study was to evaluate the renal toxicity of CTFE and HFP, several methods of investigating anatomical and functional changes within the kidney were examined. Among these were measurements of water intake, urine osmolality, blood urea nitrogen (BUN), serum creatinine, urinary glucose, and urinary and serum enzymes.

A method of evaluating renal dysfunction was developed by Radford (2). Radford demonstrated that when rats were given free access to water, they voluntarily restricted their intake and excreted an almost maximally concentrated urine. When renal function was upset, water intake and urine volume increased while solute concentration decreased. Thus, it has been demonstrated that practical surveillance of renal function is possible by measuring water intake and urine concentration of the exposed rats.

Urinary enzyme determinations as indices of renal damage have a distinct advantage over histologic studies in that they are non-invasive and may be repeated on the same animal indefinitely, thus allowing a time course study without involving individual animal variations (12-18). In the present study, the usefulness of urinary lactic dehydrogenase (LDH) and alkaline phosphatase (ALP) was investigated with particular interest in detection of early subtle injury.

Increases in urinary LDH activities have been noted following renal insult (12-15). It was first suggested that urinary LDH is renal in origin by Rosalki and Wilkinson (12). Previously, it had been shown that increases in urinary LDH activities accompanied proteinuria (13). It was then believed that the increased urinary LDH was of serum origin. However, Rosalki and Wilkinson demonstrated that animals with elevated serum LDH activities following myocardial infarction did not excrete increased amounts of LDH into the urine. Conversely, they found increased urinary LDH activities in humans with renal damage. In 1967 Plummer and Leathwood (14) demonstrated via electrophoretic separation and characterization of the five LDH isoenzymes that the urinary LDH found

after renal insult matched those of the kidney, and not those of the ureters or bladder.

In 1978 Bhargava, Khater, and Gunzel (15) found that an increase in urinary and serum LDH activity accompanied renal damage induced in rats by intravenous injection of mercuric chloride. Bhargava et al. reported a positive correlation between the enzyme activity and the dose given. In addition, the enzyme activity positively correlated with the amount of renal damage demonstrated by morphological and enzyme-histochemical methods.

Another enzyme located in the kidney that has been measured as an index of renal toxicity is alkaline phosphatase (ALP) (16-18). The brush borders of the proximal convoluted tubules have been found to be rich in ALP (19,20).

Increases in urinary ALP were first related to renal toxicity by Breedis, Florey and Furth (16), who injected concentrations of uranium nitrate into rats and rabbits. They then discovered significant increases in the total urinary concentration of ALP. Since the brush borders along the proximal tubules of the kidney have large amounts of this enzyme, while the distal convoluted tubules (19,20), collecting ducts (19,20) and ascending limb of the loop of Henle have none (21), it would seem that increased urinary ALP activities would indicate brush border damage. Electron microscopy has revealed brush border damage of the proximal tubules as one of the earliest results of subtle renal damage (22-24). Thus far however, no studies have clearly demonstrated that urinary activities of brush border enzymes, including ALP, are any more sensitive

than other indices for detection of early sublethal renal injuries,,
although dose related increases in the urine have been found (18)..

STATEMENT OF THE PROBLEM

The purpose of this study was to provide a comparative characterization between the renal toxicities of CTFE and HFP.

Since CTFE is an industrial chemical known to be toxic to rat kidneys, and to which workers could be accidentally exposed, it was chosen for this study. HFP, a pyrolysis product of polytetrafluoroethylene and thought to be one of the toxins involved in polymer fume fever, has also been found to be renal toxic and was therefore chosen for a comparative study with CTFE.

In lieu of existing evidence that CTFE and HFP are renal toxic, we considered it important to evaluate the post-exposure events in detail. Of particular interest were the time courses of renal functional and morphological disorders, that is, how rapidly the disorders, and recovery from them, occurred. When possible, correlations were drawn between the time course of morphological and functional changes and the concentration of fluoroalkene to which rats were exposed.

By characterizing the acute renal toxicities of these compounds in rats, it was anticipated that guidelines could then be established for studying the effects of chronic low level exposures from which allowable limits of human exposure in the work place may ultimately be prescribed.

MATERIALS AND METHODS

Animals

Fischer-344 rats were chosen for the study because they have been shown to be more sensitive to renal toxicity than other strains (25).

Male rats (Charles Rivers) weighing 200-275 grams were randomly divided into groups of 10 and placed in stainless steel metabolism cages. The rats were housed in these cages for at least 5 days prior to exposure so they could adapt to these surroundings. Food and water were allowed ad libitum. Rats were ordered in numbers large enough to complete whole sections of each study.

Chemicals

CTFE (Matheson, Cucamonga, Ca.) and HFP (Peninsular Chem. Research, Gainesville, Fla.) were analyzed for purity using a Finnigan 9500 gas chromatograph with Poropak Q stainless steel column interfaced with a Finnigan 3300 mass spectrometer with Data System 610. HFP was found to be 100% pure. CTFE was found to be 99% pure. The only contaminant was determined to be trifluoroethylene.

CTFE and HFP certified standard mixtures, 326 and 524 ppm CTFE, and 529 and 3102 ppm HFP with balance of air, were obtained from Matheson.

All chemicals used in the assays were of reagent grade.

Exposure System

Chamber

The exposure system consisted of a 34.0 liter pyrex cylinder (Fig. 1) with a face plate (Fig. 2) constructed of wood with a steel plate toward the inside of the chamber. At the periphery of the face plate was a 1 inch wide strip of soft porous rubber which formed an interface with the rim of the cylinder. The face plate was equipped with an intake, exhaust, and a sampling port. The intake was constructed of copper tubing.

Delivery System

The delivery system for CTFE consisted of a syringe-drive infusion pump (Harvard Apparatus Compact Infusion Pump) with two 50cc glass syringes which were filled from a size 3A cylinder containing the compressed CTFE (100 psi) via a needle valve. The 3-way stopcocks were then adjusted such that the route to the CTFE cylinder was closed and the route to the chamber was open. The CTFE was delivered by the infusion pump into a tygon hose carrying air released from a compressed air cylinder (3,000 psi). The CTFE was injected via hypodermic needles into the air hose at a flow rate determined by the particular setting of the infusion pump. The mixture entered the exposure cylinder through the intake tube. The desired concentration of CTFE for the exposure was obtained by manipulating the flow rates of the CTFE and air. The air flow rate was 10 liters/minute, which would require a CTFE flow of 1.0 ml/minute to achieve a chamber concentration of 100 ppm CTFE.

CHAMBER

Figure 1. Diagram of 34.0 liter pyrex exposure chamber

FACE PLATE

Figure 2. Diagram representing the face plate of the exposure chamber

The required rate of delivery of HFP exceeded that practical by the infusion pump. Therefore, the HFP was delivered directly from its pressurized cylinder (100 psi) into the air hose through the hypodermic needles. The flow of HFP was monitored by a gas flow meter, and regulated by a needle valve. The air flow rates for the HFP exposure were between 10 and 15 liters/minute, with a corresponding HFP flow yielding the desired chamber concentration.

Chamber Atmospheric Analysis

The exposure chamber was constructed with 3 sampling ports, front (face plate), back, and side. Early tests indicated even distribution of fluoroalkene inside the chamber during exposures, and sampling from any port yielded the same results within 2%. Therefore, the side port was used for sampling of exposures as a matter of convenience.

Sampling was done by withdrawing a 3.0 ml atmospheric sample into an air tight syringe with a Teflon plunger.

The exposure atmosphere of the chamber was analyzed by gas chromatography on Porapak Q (4mm x 6 ft. stainless steel column) using a Varian 3700 Gas Chromatograph equipped with a flame ionization detector and Varian CDS 111 integrator. Column temperature = 150° C, injection port temperature = 180° C, detector temperature = 180° C, and carrier flow = 30.2 cc/minute. Using the flame ionization detector the fluoroalkene peak was the only peak to appear.

The samples were analyzed within 3 or 4 minutes as it was found that the fluoroalkene concentration, within the gas tight syringe, diminished with time, probably due to diffusion into the Teflon plunger.

Exposures

The exposures were generally performed between 10:00 A.M. and 2:00 P.M. in order to avoid diurnal variations. Atmospheric samples were taken after 5, 15, and 30 minutes, and every 30 minutes thereafter for the remainder of the exposure. If the concentration had not stabilized after the first 30 minutes, samples were taken every 10 minutes until a steady state concentration was achieved. The fluoro-alkene flow was stopped 3 hours and 55 minutes into the exposure, an atmospheric sample taken, and the chamber purged. At the 4.0 hour mark, the final sample was drawn, and the rats were immediately removed from the chamber and placed back into the metabolism cages to start the observation period.

Urine Analysis

Collection and Dialysis

Twenty-four hour urine samples were collected for 2 rats in plastic cups, measured for volume, placed in plastic tubes, centrifuged, and dialyzed against distilled water for 80 to 90 minutes. Samples were collected in plastic containers, as opposed to glass, to prevent loss of urinary F^- which would adhere to glass. The stainless steel metabolism cages did not allow for collection of urine free of fecal contamination. Samples in the HFP study contained 50 microliters of streptomycin (10 mg/ml) to prevent possible bacterial growth due to this contamination. Samples in the CTFE study contained no antibiotics.

Dialysis Procedure:

1. 5.0 ml of centrifuged urine were pipetted into a cellulose dialysis bag and immersed in distilled water with a rinsing flow of 5.0 ml per minute.
2. After 80 to 90 minutes, the dialyzed urine was poured into a graduated cylinder and the final volume recorded.
3. Using the final volume, the dialysis dilution factor (DDF) was calculated as:

$$\text{DDF} = \frac{\text{Final Volume (ml)}}{5.0 \text{ ml}}$$

Osmometry

Daily osmolalities were measured by freezing point depression osmometry (Advanced Osmometer, Advanced Instruments, Inc., Newton Highlands, Mass.) using 1.0 ml of nondialyzed urine mixed with 1.0 ml of water. The osmometer reading was then multiplied by a factor of 2. This method was preferred over measuring the osmolality of undiluted urine because it was faster (less cooling required with lower concentration), and it conserved urine for other assays, though it decreased the sensitivity of the osmometry. The osmometer was calibrated with standard solutions of 200, 500, and 900 milliosmols (mOsm)/kg of solution, which were obtained from Advanced Instruments, Inc.

Urinary Fluoride

Daily urinary fluoride levels were measured on days 0 (day of exposure) through 4 after exposure. Levels were measured with a Corning

Digital 110 Expanded Scale pH Meter with an Orion Research Model 605 electrode switch.

Procedure:

1. Standard solutions were prepared with fluoride concentrations ranging from 0.001 to 1.0 micromol/ml.
2. 1.0 ml of each standard and 24 hour sample of nondialyzed urine was mixed with 1.0 ml TISAB buffer.
3. The fluoride electrode was immersed in each standard and sample, and allowed to equilibrate for 5 minutes, after which the millivolt (mv) readings were taken.
4. The mv readings for the standards were plotted vs the log of the concentration, and a regression analysis was performed. From the regression analysis the concentration of each of the samples were determined as micromol F^- /ml urine.
5. The amount of fluoride excreted for 24 hours was calculated as follows:

$$\mu\text{m } F^- / 24 \text{ hours} = \mu\text{m } F^- / \text{ml urine} \times \text{ml urine} / 24 \text{ hours}$$

Urinary Glucose

Urinary glucose levels were measured on days 0 through 5 after exposure to HFP. The glucose oxidase method (26) with deproteinization was used for the assays.

Principle:

The brown oxidized o-dianisidine absorbs light at 425 nm. The concentration of glucose is then proportional to the increase in absorbance at 425 nm.

Reagents:

Barium hydroxide (0.3 N)

Zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5%)

Glucose standard (1.0 mg/ml)

Glucose oxidase

o-dianisidine dihydrochloride

Combined enzyme-color reagent: 1.6 ml of o-dianisidine dihydrochloride (2.5 mg/ml) was added to 100 ml of glucose oxidase solution (5 International Units/ml), and mixed by inversion.

Procedure:

The procedure was performed per Sigma bulletin No. 510 UV (Sigma Chemical Co., St. Louis, Mo.).

Urinary LDH

Urinary LDH activities were determined using a modification of the Sigma LDH assay procedure, using the Sigma LDH Determination Kit No. 340-UV.

Principle:

NADH has highest light absorbance at 340 nm; NAD^+ has low absorbance at 340 nm. Therefore, the reaction velocity is measured as the decrease in absorbance units per minute at 340 nm.

Reagents:

NADH (0.2 mg/assay)

pyruvate

potassium phosphate buffer (0.1 M, pH 7.5)

Procedure:

The procedure was performed per Sigma bulletin, noted above, with the following modification: 200 μ l urine were used instead of 50 μ l which were called for.

The number of LDH units¹ excreted per 24 hours were calculated as:

$$\text{Units LDH/24 hour} = \text{Units LDH/ml urine} \times \text{ml urine/24 hours}$$

Urinary ALP

Urinary ALP activities were determined using a modification of the Sigma Phosphatase assay Kit No. 104.

Principle:

The reaction is allowed to proceed in a solution of pH 10.5. The activity of the ALP present is proportional to absorbance at 400-420 nm of the yellow p-nitrophenol formed.

1. The units calculated represent a decrease in A(340) of 0.001 per minute, and may be converted to International Units (IU) by multiplying by a factor of 0.48.

Reagents:

a) Phosphatase substrate: 100 mg p-nitrophenyl phosphate disodium was mixed with 25.0 ml water. 2.0 ml aliquots were dispensed into vials, stoppered, and frozen upright. These preparations were stable for 6 weeks.

b) Alkaline buffer solution: glycine 0.1 M in magnesium chloride 0.001 M, pH 10.5. Chloroform was added as a preservative. The solution was stored at 0.5°C.

Procedure:

The procedure was performed per Sigma Bulletin noted above, with the following modification: 0.1 ml dialyzed urine were used instead of the 2.0 ml called for.

The number of ALP units² excreted per 24 hour period were calculated as:

$$\text{Units SLP/24 hours} = \text{units/ml urine} \times \text{ml urine/24 hours}$$

Blood AnalysisSerum Creatinine

Serum creatinine assays were performed with the use of an automatic analyzer (Technicon AutoAnalyzer). The method employed was a modification of the procedure of Folin and Wu (27).

2. The units calculated were Sigma units, which may be converted to International Units (IU)/L by multiplying by a factor of 16.7

Reagents:

Saline composed of sodium chloride (9.0 grams) in 1.0 liter of distilled water

Sodium hydroxide, 0.5 N

Saturated picric acid solution consisting of 13 gm picric acid in 1.0 liter of distilled water

Stock creatinine standards of concentrations ranging from 1-10 mg creatinine per 100 ml solution of 0.2 N HCl

Procedure:

The procedure is fully automated, and 60 determinations per hour could be accomplished.

The creatinine concentrations were read directly from the chart paper by peak height, giving values in mg creatinine per 100 ml serum (mg%).

BUN

The BUN values were also obtained from the Technicon AutoAnalyzer. The procedure was a modification of the carbamido-diacetyl reaction as applied to the determination of urea nitrogen (28,29). It is based on the direct reaction of urea and diacetyl monoxime (2,3-butanedione-2-oxime) under strongly acidic conditions. Diacetyl monoxime is hydrolyzed to diacetyl in acid solution. The diacetyl then reacts with urea, in the presence of an acidic ferric alum reagent to form triazine derivatives by an oxidative condensation reaction. The product of the reaction absorbs light at 480 nm, thus allowing reaction measurement.

Urea nitrogen can be determined in whole blood, serum, plasma, cerebrospinal fluid, or urine. In these studies determinations were made using whole blood.

Reagents:

Saline (same as used in the serum creatinine procedure)

Ferric alum-acid: 500 gm of ferric ammonium sulfate [$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] were dissolved in 500 ml of distilled water, 500 ml o-phosphoric acid (85%), and 500 ml of sulfuric acid (s.g. 1.84).

Diacetyl monoxime and urea, consisting of 10.0 ml urea nitrogen standard (100 mg%) in 1.0 liter of diacetyl monoxime

Standards:

Standard diluent: 0.20 gm phe-ylmercuric acetate and 1.4 ml concentrated sulfuric acid were mixed with 5.0 liters of distilled water.

Stock urea nitrogen (10.0 mg urea N/ml); 21.433 gm urea were mixed with 1.0 liter standard diluent.

Working urea nitrogen standards: the stock urea nitrogen standard was diluted with standard diluent to achieve the following concentrations, 10.0, 30.0, 50.0, 70.0, 100.0, and 150.0 mg%.

Procedure:

The procedure was fully automated, and BUN concentrations were read directly from the chart paper as a function of peak height, yielding values of BUN in mg%.

The kidney samples from rats exposed to CTFE were dehydrated and imbedded in paraffin and sectioned by routine methods. Sections were stained with hemotoxylin and eosin (H&E). Sections from HFP exposed animals were stained using periodic acid Schiff (PAS) stain. The PAS was preferred as it allowed better visualization of the renal architecture.

Statistics

Tests for significance of difference between two means were performed using Student's t test. Tests for significance of correlation were performed using the Pearson Product Moment Correlation and table of critical values.

RESULTS

Rats were exposed to 100 (102 ± 16.3), 220 (222 ± 36.9), 330 (330 ± 66.4), 540 (544 ± 24.4) ppm CTFE, and 380 (380 ± 27.0), 470 (467 ± 72.0), 660 (660 ± 191.0), and 1200 (1188 ± 60.0) ppm HFP. The variation in chamber concentration during exposures was around 16% with a range of 4.5% to 30% (Figs. 3,4). The chamber concentration usually reached to within 60% of the anticipated exposure concentration within 5 minutes, and generally stabilized within 30 minutes. Air flow was 10 liters/minute during the CTFE exposures. Higher air flow rates were sometimes required during the HFP exposures. Since it was difficult to control the delivery rate of HFP with the needle valve at lower concentrations, a higher flow was necessary, thus necessitating a higher air flow rate to achieve the desired concentration.

Exposure System

All exposures were inside the 34 liter pyrex cylinder as described in the Materials and Methods section. This chamber was found to be quite adequate for this study. Ten rats were accommodated without obvious overcrowding.

An air flow rate of 10.0 liters/minute was found to prevent moisture condensation on cylinder walls when 10 rats were inside the chamber.

Figure 3. Chamber atmospheric concentrations during 4.0 hour exposures of rats to CTFE -- Curve 1 represents 100 ppm ($101.8 \text{ ppm} \pm 16.0\%$); Curve 2, 220 ppm ($222.1 \text{ ppm} \pm 16.6\%$); Curve 3, 330 ppm ($329.8 \text{ ppm} \pm 20.1\%$); and Curve 4, 540 ppm ($543.6 \pm 4.5\%$).

Figure 4. Chamber atmospheric concentrations during 4.0 hour exposures of rats to HFP -- Curve 1 represents 380 ppm (379.2 ppm $\pm$ 7.1%); Curve 2, 470 ppm (467.0 ppm $\pm$ 15.4%); Curve 3, 650 ppm (659.3 ppm $\pm$ 30.0%); and Curve 4, 1200 ppm (1188.5 ppm $\pm$ 5.0%).

The delivery system used in the CTFE study consisted of an infusion pump with two 50 cc glass syringes as described in the Materials and Methods section. This system was found to be adequate, for this study, but complicated to use, and difficult to control. The syringes required frequent refilling with CTFE (the higher the exposure concentration, the more frequent the refilling; the 500 ppm CTFE exposure required refilling approximately every 15 minutes), which induced variability in the rate of infusion of CTFE into the chamber. This variability arose because the syringes were loaded directly from the CTFE pressurized cylinder by allowing pressure from the cylinder to drive the syringe plungers back. Thus, there was a residual positive pressure left in the syringes after filling. To alleviate this pressure, the plungers were pulled back manually with all routes away from the syringes closed. When this manual adjustment was finished, unless the pressure inside the syringes equalled the pressure of the exposure atmosphere, either a positive or negative pressure was likely to exist when the route from the syringes to the chamber was opened. In either case an abrupt deviation from the desired CTFE flow rate ensued. It was found that, since relatively low flow rates were desired, best results were obtained by manually pulling the plungers back, after refilling, such that a negative pressure resulted inside the syringes. A temporary slight drop in chamber CTFE concentration may have resulted, but this change was observed to cause a smaller deviation in the exposure concentration, and thus was the preferred method.

HFP was delivered directly from the pressurized cylinder to the exposure chamber via a needle valve. This system was much less

cumbersome, as there were no syringes to be filled during the exposure. As long as correct flow of HFP was maintained, no more manipulating of the delivery system was required, and no large deviation in flow of HFP was observed. The problematic area in this system was the needle valve. At flow rates of HFP required (5.0 to 18.0 ml/min), the needle valve was only slightly opened, making it difficult to maintain a steady, uniform flow. Thus, deviation in flow rate of HFP resulted, particularly at lower concentrations.

Body Weight

Following exposure to CTFE and HFP, rats exhibited decreases in the rate of body weight gain (Figs. 5, 6). Though these decreases were not well correlated to concentration, they were greatest in rats exposed to the highest concentrations of fluoroalkene. Experimental control rats employed lost more weight than most of the fluoroalkene exposed rats. This result was unexpected and it is unknown why it occurred. Control rats were exposed to air for 4.0 hours in the same chamber as rats receiving fluoroalkene, were housed on the same cage rack as exposed animals, and received the same amount of handling. Control rats were housed on the bottom row of the cage rack. Therefore, if they were not to have received food, it would easily have been noticed. In Figures 5 and 6 the expected weight gain of control animals is plotted. This line was derived from a chart, provided by Charles Rivers, of recorded weight gains of Fischer-344 male rats between the weights of 205 to 255 grams over a two week period.

Figure 5. Daily increases in body weight following exposures to CTFE -- Following single 4.0 hour exposures to CTFE, rats exhibited decreases in the rate of weight gain. All weights were normalized to 0 on Day 0 to equate weight changes before and after exposure. (*) Rate of body weight gain for Fischer-344 rats between 205 and 255 grams (from growth chart published by Charles Rivers).

Figure 6. Daily increases in body weight following exposures to HFP -- Following single 4.0 hour exposures to HFP, rats exhibited decreases in the rate of weight gain. All weights were normalized to 0 on Day 0 to equate weight changes before and after exposure. (*) Rate of body weight gain for Fischer-344 rats between 205 and 255 grams (from growth chart published by Charles Rivers).

Kidney Weight

Dose related increases in kidney weights were noted following exposure to CTFE or HFP (Figs. 7, 8). None of the rats exposed to CTFE had significantly elevated kidney weights on day 1, but rats exposed to 380 and 470 ppm HFP did ($p < 0.05$). The control value determined for this study was the mean of seven kidney weights in rats exposed for 4.0 hours to air only. To determine the dose response relationship, values for the first 3 days following exposure were averaged for CTFE exposed rats, and values for the first 4 days were averaged for HFP rats. These mean values were then plotted versus concentration of fluoroalkene (Fig. 9). It was found that kidney weight varied linearly with concentration of fluoroalkene rather than log of the concentration. This was unexpected, and may be due to sampling error.

Urine Osmolality

Decreases in urine osmolality occurred following exposures to CTFE and HFP (Figs. 10, 11) at all concentrations except 100 ppm CTFE. Rats exposed to 220, 330, and 540 ppm CTFE exhibited significant decreases in urine concentration for 3 days post-exposure, with the 540 ppm group showing decreases for 4 days. All groups exposed to HFP exhibited significant decreases for 4 days after exposure. The 1200 ppm HFP group did not excrete urine of significantly depressed osmolality on day 1, but the values for the other post-exposure days were significantly depressed.

Figure 12 shows decreasing urine osmolality versus the log of the concentration of fluoroalkene. The correlation coefficients for the CTFE

Figure 7. Daily kidney weights in rats following single 4.0 hour exposures to CTFE -- Values represent the mean of two weights.

Figure 8. Daily kidney weights in rats following single 4.0 hour exposures to HFP -- Values represent the mean of two weights.

Figure 9. Kidney weights of rats versus concentration following single 4.0 hour exposures to CTFE or HFP -- Values represent mean and standard deviation of values averaged over the first 3 days for CTFE, and the first 4 days for HFP.

Figure 10. Daily urine osmolalities following single 4.0 hour exposures of rats to CTFE.

Figure 11. Daily urine osmolalities following single 4.0 hour exposures of rats to HFP -- Values represent mean plus and minus standard deviation.

Figure 12. Urine osmolality versus log of the concentration following single 4.0 hour exposures to CTFE and HFP -- Values represent mean plus and minus standard deviation.

and HFP curves were -0.7598 and -0.7805, respectively. Though these correlations are nonsignificant ($p > 0.10$), they do indicate that urine osmolality tended to decrease with increasing concentration of HFP and CTFE to which rats were exposed.

Water Intake

Water intake was determined on a daily basis before and after exposure. Polydypsia was noted following exposure to all concentrations of CTFE and HFP (Figs. 13, 14). On day 1 all groups exhibited dose related increases in their water intake. Significant increases were noted through day 3 for all groups except the 100 ppm CTFE group, which had returned to pre-exposure levels of intake and remained within normal limits thereafter (6). Intake for the CTFE groups reached maxima on Day 2 with all groups to control levels by day 6. Times at which maxima occurred varied in HFP exposed rats. Groups exposed to 380, 470, and 660 ppm consumed maximum levels within 24 hours, and continued increased consumption through day 3. The group exposed to 1200 ppm HFP reached maximum intake on day 3. Rats exposed to HFP exhibited increased water intake for longer periods of time than did rats exposed to CTFE.

Figure 15 represents increases in water intake after exposure versus log of the concentration of fluoroalkene to which rats were exposed. The best fit obtained for the dose-response of water intake was a semi-log fit, suggesting a sigmoidal shaped curve if water intake values were plotted versus concentrations ranging from zero to the

LC₉₉.

Figure 13. Daily water intake by rats following single 4.0 hour exposures to CTFE

Figure 14. Daily water intake by rats following single 4.0 hour exposures to HFP -- Values represent mean plus and minus standard deviation.

Figure 15. Changes in water intake versus log of the concentration following single 4.0 hour exposures to CTFE and HFP -- Values represent mean plus and minus standard deviation.

CTFE appears the more potent toxin, but HFP produced a curve with a greater slope (38.6 vs 23.4). The curves intersect at 2.87 on the log scale, 741 ppm. This suggests that, although CTFE is a more potent renal toxin, HFP may be capable of inflicting more damage at higher doses.

Urinary Fluoride

Large increases in urinary F^- (a probably metabolite of CTFE and HFP) were noted in the first 24 hours following exposure to CTFE and HFP, but returned to control levels on day 2 (Figs. 16, 17). Control values for this experiment were determined by averaging pre-exposure levels. These values were $1.66 \pm 1.05 \mu M F^- / \text{rat} \cdot 24 \text{ hours}$ ($N=12$), for CTFE, and $2.6 \pm 0.3 \mu M F^- / \text{rat} \cdot 24 \text{ hours}$ ($N=6$) for HFP exposed rats. Increased levels of urinary F^- were found to correlate linearly to the concentration of fluoroalkene. Figure 18 shows the dose-response relationship of urinary F^- levels for CTFE and HFP. The best fit for the dose-response curve was obtained using concentration of fluoroalkene, as opposed to log of the concentration. This was expected since there should not be a threshold requirement for metabolism. It appeared that CTFE and HFP underwent metabolism (such that similar amounts of F^- were generated). The curve is linear up to about 600 ppm fluoroalkene. Above that concentration, the values for HFP leveled off, suggesting possible renal shutdown, and therefore limited inorganic F^- excretion. The value for the 1200 ppm HFP group was not considered in the regression analysis.

Figure 16. Daily levels of urinary fluoride excretions following single 4.0 hour exposures of rats to CTFE

Figure 17. Daily levels of urinary fluoride excretion following single 4.0 hour exposures of rats to HFP

Figure 18. Maximum levels of inorganic fluoride excretions versus concentration of fluoroalkene, following single 4.0 hour exposures of rats to CTFE or HFP

BUN

Following exposure to CTFE and HFP, rats exhibited dose related increases in BUN (Fig. 19). Increases in BUN occurred at concentrations of CTFE lower than those of HFP, which is consistent with other data indicating CTFE to be a more potent renal toxin than HFP. The slopes of the curves obtained were very similar for CTFE and HFP (53.8 and 53.0, respectively). Significant correlations were obtained for both curves on semi-log plots.

Serum Creatinine

Following exposure to CTFE and HFP, rats exhibited dose related increases in serum creatinine (Figs. 20, 21). Rats exposed to CTFE had elevated levels of serum creatinine on days 1 and 2 at all concentrations of CTFE above 100 ppm (all values of $p < 0.001$, except when $N=1$; then the values were more than 3 standard deviations above the control value, and therefore may be considered elevated). The value obtained from control rats was 0.61 ± 0.08 mg% ($N=9$). Dose related increases in serum creatinine were noted on days 1 through 5 in rats exposed to HFP. The value for the 470 ppm group (1.0 mg%, $N=1$) on day 1 was more than 3 standard deviations above control. The highest values found in rats exposed to HFP were on days 2 or 3. The highest values found in CTFE exposed rats occurred on day 2 for all groups showing significant increases, though the values were only slightly above those found on Day 1. Creatinine levels of the 540 ppm CTFE group were the only values still elevated on day 3 with all values normal by day 8. Since rats

Figure 19. Maximum BUN values versus log of the concentration for rats following single 4.0 hour exposures to CTFE or HFP -- Values represent mean plus and minus standard deviation.

Figure 20. Daily levels of serum creatinine in rats following single 4.0 hours exposures to CTFE -- Values represent mean plus and minus standard deviation.

Figure 21. Daily levels of serum creatinine in rats following single 4.0 hour exposures to HFP -- Values represent mean plus and minus standard deviation.

were only observed for 5 days post-exposure to HFP, it is unknown how long the levels would have been elevated.

Figure 22 shows the semi-log dose-response curve for the serum creatinine levels in rats exposed to CTFE and HFP. CTFE exposed rats exhibited serum creatinine levels significantly correlated to log of the concentration ($r=0.9981$, $p < 0.01$). Though HFP exposed rats did not have serum creatinine increases significantly correlated to concentration ($r=0.8889$, $p > 0.10$); the levels tended to increase with increasing concentration.

Urinary Glucose

As a further consideration in the HFP study, urinary glucose levels were measured. Rats exposed to 380, 470, and 1200 ppm HFP exhibited elevated urinary glucose levels for at least 3 days (Fig. 23). Values for rats exposed to 660 ppm HFP were not determined. By day 4, values for the 380 ppm group had returned to control levels; values for the 470 and 1200 ppm groups remained significantly elevated. The control value for the experiment was obtained by averaging pre-exposure values for the rats: control = 1.6 ± 0.9 mg/rat·24 hours (N=8).

Urinary LDH

Rats exhibited dose related increases in urinary LDH excretions within 24 hours following exposure to CTFE or HFP (Figs. 24, 25). Rats exposed to 470 and 660 ppm HFP excreted maximum levels of LDH on day 1, but the 380 and 1200 ppm HFP groups excreted maximum amounts on day 2 following exposure. Control values employed were the mean of

Figure 22. Maximum serum creatinine levels versus log of the concentration following single 4.0 hour exposures to CTFE or HFP -- Values represent mean plus and minus standard deviation.

Figure 23. Daily urinary glucose excretion levels from rats following single 4.0 hour exposures to HFP -- Values represent mean plus and minus standard deviation.

Figure 24. Daily levels of urinary LDH excretions by rats following single 4.0 hour exposures to CTFE -- Values represent mean plus and minus standard deviation.

Figure 25. Daily levels of urinary LDH excretions by rats following single 4.0 hour exposures to HFP -- Values represent mean plus and minus standard deviation.

pre-exposure values for the CTFE and HFP groups and were determined to be 132.7 ± 114.0 and 51.8 ± 40.5 units/rat·24 hours, respectively. All groups exhibited significantly elevated levels of urinary LDH on day 1 except the group exposed to 100 ppm CTFE. All LDH activities from rats exposed to CTFE returned to control levels by day 4. Groups exposed to 660 and 1200 ppm HFP continued to excrete elevated LDH activities through day 4 ($p < 0.001$). It appeared that CTFE had a more immediate effect, noting that all maximum increases occurred on day 1. Groups exposed to CTFE exhibited over a 50% decrease from maximum excretions by day 2.

Figure 26 represents increases in LDH activities versus log of the concentration of fluoroalkene. Again, CTFE appeared the more toxic compound. The lowest concentration of CTFE to produce significant increases in urinary LDH activities was 220 ppm (2.34 on the log scale). All concentrations of HFP used produced significant increases in urinary LDH. The correlation coefficients with corresponding critical values for CTFE and HFP were 0.9742 ($p < 0.05$) and 0.0047 ($p < 0.01$), respectively. The curves in Figure 26 are presented as a semi-logarithmic fit to be consistent with the presentation of other data obtained. However, when values of r were calculated using straight concentration, values for the CTFE and HFP curves were 0.9993 and 0.9974 indicating a better linear fit than semi-logarithmic fit. This probably indicates that values obtained for the concentrations employed lie on the linear portion of the sigmoidal curve expected if values were plotted for concentrations ranging from zero to the LC_{99} value.

Figure 26. Urinary LDH activities versus log of the concentration following single 4.0 hour exposures to CTFE or HFP -- Values represent mean plus and minus standard deviation.

Urinary ALP

As an additional index of toxicity, in the HFP study, urinary ALP activities were measured. Following exposure to HFP, rats excreted increased levels of ALP (Fig. 27). The control value for this experiment was determined by averaging pre-exposure levels excreted by the rats and was determined to be 0.57 ± 0.66 ($\times 10^{-3}$) units/rat $\cdot$ 24 hours (N=13). Rats exposed to 380 ppm HFP did not excrete significantly increased amounts of ALP on day 1, but did on day 2 ($p < 0.001$). At higher concentrations, rats excreted significantly increased levels of ALP on day with elevated levels persistent through day 3.

Increases in urinary ALP following exposure to HFP increased with increasing concentration, but did not increase linearly with either concentration or log of the concentration (Fig. 28). The correlation coefficient was 0.8618 ($p > 0.10$) for the semi-log plot, and 0.7922 for the linear plot ($p > 0.10$), neither representing a significant correlation. ALP activities appeared to approach a maximum after the value from rats exposed to 470 ppm HFP (2.67 on the log scale). The value of ALP for the 470 ppm group was significantly different from that of the 380 ppm group, as were values for the 660 and 1200 ppm groups. Values obtained for the 470, 660, and 1200 ppm groups did not differ significantly from each other.

Histology

Following exposure to CTFE and HFP, rats exhibited cellular necrosis of the proximal renal tubules (Figs. 29-31). Tubular necrosis was localized to the pars recta (segment P_3) of the proximal convoluted

Figure 27. Daily levels of urinary ALP excretions by rats following single 4.0 hour exposures to HFP -- Values represent mean plus and minus standard deviation.

Figure 28. Maximum units of ALP excreted by rats versus log of the concentration following single 4.0 hour exposures to HFP -- Values represent mean plus and minus standard deviation.

Figure 29. Light micrograph of a region of medullary ray of the deep cortex from a rat 1 day after a single 4.0 hour inhalation exposure to 330 ppm CTFE -- Extensive necrosis of the epithelial cells lining several pars recta (PR) portions of proximal tubels is demonstrated. H/E x 360.

Figure 29. Light micrograph of a region of medullary ray of the deep cortex from a rat 1 day after a single 4.0 hour inhalation exposure to 330 ppm CTFE

Figure 30. Light micrograph of the superficial renal cortex from a rat 2 days after a single 4.0 hour inhalation exposure to 1200 ppm HFP — Casts are seen in lumen of the distal tubules. PAS x 360.

Figure 30. Light micrograph of the superficial renal cortex from a rat 2 days after a single 4.0 hour inhalation exposure to 1200 ppm HFP

Figure 31. Light micrograph of deep renal cortex in the medullary ray region from a rat 3 days after a single 4.0 hour inhalation exposure to 300 ppm CTFE -- Necrosis of the epithelial cells lining the pars recta portion of the proximal tubule is demonstrated. Note early focal regeneration as evidenced by basophilic cytoplasm, flattened nuclei, and mitotic figure (arrows). H/E x 360.

Figure 31. Light micrograph of deep renal cortex in the medullary ray region from a rat 3 days after a single 4.0 hour inhalation exposure to 300 ppm CTFE.

tubule in CTFE exposed rats, but extended to the pars convoluta (segments P₁ and P₂) in rats exposed to the highest concentrations of HFP. In rats exposed to 1200 ppm HFP, total renal cortical necrosis was observed. The histologic changes noted, occurred within 24 hours post-exposure, and were generally most prominent within 48 hours. Regeneration was characterized by flattened nuclei, occasional mitotic figures, and slightly more basophilic cytoplasm than normal. Regeneration was usually noted by day 3. In CTFE rats, full regeneration was apparent within 2 weeks.

SGPT and SGOT

No significant changes were noted in SGPT or SGOT levels after exposure to any of the concentrations of CTFE. SGPT and SGOT were not measured in the HFP study.

DISCUSSION

CTFE and HFP are gaseous compounds encountered in the industrial setting. CTFE is a monomer used in the industrial production of the polymer polychlorotrifluoroethylene. HFP has been determined to be a pyrolysis product of polytetrafluoroethylene (6) and has been implicated in the etiology of "polymer fume fever" (7).

Since both CTFE and HFP have been shown to cause renal injury in rats (2,4,5), and therefore represent possible occupational hazards as potential renal toxins, we felt it important to investigate their renal toxicities in rats. Fischer-344 rats were chosen since they have been shown to be more sensitive to renal injury than other strains (26).

The most sensitive index of toxicity with respect to early damage was diuresis as revealed by increased water intake and decreased urine osmolality. That CTFE and HFP produced diuresis almost immediately following exposure may reflect an inhibition of solute reabsorption in the kidney tubules, thereby inhibiting reuptake of water.

Measurements of water intake and urine osmolality proved to be very practical indices of renal function. Since they were noninvasive measurements, they allowed for time course studies of renal concentrating ability of the kidneys preceding and following exposure to fluoroalkenes, and have proven to be valuable for the study of the chronology of events following exposure.

The dose related increases in serum creatinine and BUN levels indicate that CTFE and HFP reduced plasma clearance ability of kidneys in rats. In agreement with changes in daily water intake and urine osmolality, dose response curves for serum creatinine and BUN indicated that CTFE was a more potent renal toxin than HFP.

Since all serum creatinine values reached maxima within 2 days following exposure to CTFE, as did the levels of diuresis, it appears that maximum impairment of renal function occurred earlier in the rats exposed to CTFE than to HFP, which often exhibited maximum dysfunction on day 3.

Increases in urinary glucose excretion following exposure to HFP also indicated that maximum renal dysfunction occurred on days 2 or 3 in these rats. Increases in urinary glucose levels probably indicate a decrease in hexose transport in the tubules following toxic insult. Another possible explanation for the glucosuria could be elevated serum levels of glucose such that the tubular maximum for hexose transport was exceeded; serum glucose levels were not measured in this study. Since inorganic F^- has been shown to result in increased serum glucose levels in rats (30), the possibility then exists that inorganic F^- generated during metabolism of fluoroalkenes may have had a role in these findings, though it is unknown what sorts of serum levels of F^- were attained following the exposures. Serum F^- levels were not measured as we attempted to obtain maximum data from the animals in a noninvasive manner.

Urinary lactic dehydrogenase was measured since it has been shown to be a highly sensitive indicator of proximal tubular necrosis (14, 15). Dose related increases in urinary LDH indicated dose related proximal tubular necrosis following exposure to CTFE or HFP. Since increased serum LDH has been shown not to result in increased urinary levels of LDH (12), these increases could only have resulted from necrotized cells of the proximal tubules. Furthermore, increases in urinary LDH activities were observed only in those rats exposed to concentrations of fluoroalkene high enough to produce necrosis, thus offering a positive correlation between increases in urinary LDH and necrosis of the proximal tubule of the rat kidney.

Since urinary ALP has been found in greatest amounts in brush borders of proximal tubules of the kidney in rats (31,32), and since early renal damage has been shown histologically to result in loss of brush border (21, 23), the increases in urinary ALP, noted following exposure to HFP, indicate early damage of the brush border of the proximal tubule.

The dose response curve for urinary ALP versus log concentration of HFP revealed what appeared as a maximum effect suggesting that low toxic concentrations of HFP were capable of inflicting almost maximal brush border damage, yielding almost maximum urinary ALP excretions.

These findings indicate that urinary enzymes are useful indicators of early morphological changes within the kidney and may be substituted for procedures such as histology and histochemistry which usually require sacrificing the animal. When employed in combination

with measurements of daily water intake and urine osmolality, it appears that urinary enzyme determinations offer a practical system for researchers to monitor the time course of toxicity with respect to functional impairment and certain morphological alterations of the proximal tubule of the kidney following toxic insult.

In histologic examination of the kidneys, frank necrosis of the proximal tubules was noted. Since the cellular damage noted was destruction of epithelial cells, but not basement membrane, rapid regeneration followed the damage, and was often apparent within 3 days following exposure. As CTFE exposed rats exhibited full recovery of renal function and regeneration of damaged cells within 2 weeks, it appears that the damage inflicted was largely reversible at the concentrations to which rats were exposed.

It appeared that metabolism of HFP and CTFE was practically complete within 24 hours after exposure as urinary inorganic F^- levels returned to normal by day 2. Though urinary F^- excretions appeared to approach a maximum after exposure to 650 ppm, it did not appear that any unmetabolized HFP underwent degradation on day 2 following exposure to 1200 ppm HFP. This suggests that the parent HFP was removed from sites of metabolism either by the kidney, assuming it was not metabolized to a great extent in the kidney, or by sequestration elsewhere in the body. Dilley (4) found cyclic F^- excretions in rats following exposure to HFP. Increased F^- excretions ran in cycles of about 5 days, suggesting that either HFP or inorganic F^- was being sequestered in the body.

In our study, the amounts of F^- excreted were linear with respect to dose until the exposure concentration of HFP exceeded 650 ppm. If F^- were being sequestered by the body, thereby keeping it out of the urine, one would expect constant low level urinary F^- excretions until a certain threshold serum concentration was attained, at which time dose dependent F^- excretions would appear. Since we observed the opposite, it is suggested that inorganic F^- was not sequestered. Therefore, the parent HFP which was not metabolized within 24 hours was probably removed from the serum unchanged, and either stored somewhere in the body or excreted.

It is unclear whether the primary toxic agent involved in renal damage was the parent compound, a bioactivated intermediate, or a toxic conjugate formed during phase II metabolism. Though inorganic F^- has been shown to induce diuresis in rats, it seems unlikely that inorganic F^- generated during metabolism was the primary toxic agent responsible for the diuresis noted following exposure to CTFE or HFP. Had inorganic F^- been solely responsible for the tubular damage and resulting diuresis, a dose response relationship would be expected between diuresis and the amount of F^- excreted into the urine. This relationship does not hold when comparing results of exposure to different compounds.

Cousins et al. (33) performed a study in male Fischer rats in which diuresis was induced by the fluorinated anesthetic methoxyflurane. They determined inorganic F^- generated during metabolism to be the primary toxic agent. However, in order for their rats to exhibit

decreased urine concentrating ability comparable to that of our rats, they had to achieve body levels of F^- such that they excreted nearly $40 \mu M F^-$ /24 hour period, 4 times the amount excreted by our rats. This casts some doubt as to whether inorganic F^- generated during metabolism was the primary toxic agent involved in fluoroalkene induced diuresis. Furthermore, the amount of inorganic F^- excretion was proportional to the concentration of fluoroalkene; that is, the same amount of urinary F^- was excreted per 24 hour period, at equal concentrations of CTFE and HFP. If inorganic F^- were the primary toxic agent, we would then expect the same dose-response relationship for CTFE as for HFP. This did not appear to be the case however, since CTFE was found to be more potent than HFP. Therefore, a toxin other than inorganic F^- is indicated.

A possible toxic conjugate of CTFE could be formed as trifluorovinylcysteine in a reaction analogous to the formation of dichlorovinylcysteine, which has been found to be a renal toxin resulting from trichloroethylene (34). Trifluorovinyl-L-cysteine has been synthesized by Gandolfi and found to cause extensive renal necrosis in mice (35). Since conjugation reactions are generally thought to be detoxifying mechanisms, the possibility of formation of a highly toxic conjugate becomes of great toxicological interest, and may be an important link in the etiology of renal toxicity of halogenated alkenes as well as other compounds.

While the mechanism by which CTFE and HFP cause necrosis is unknown, neither is it clear why cells of the pars recta appear to be

damaged before cells of the pars convoluta. An obvious postulate to explain apparent susceptibility of cells of the pars recta to this toxicity is that the region of the pars recta is subjected to higher concentrations before the region of the pars convoluta. When the toxin is introduced into the pars convoluta portion of the proximal convoluted tubule in the glomerular filtrate, it is diluted to less than threshold concentration required to induce necrosis. But, as over 50% of the water of the filtrate is reabsorbed in the pars convoluta (36), the toxin becomes more concentrated as it passes along the tubule until threshold concentration is attained in the pars recta resulting in toxicity to these cells. Histology of kidneys from rats exposed to the lowest concentration of HFP revealed necrosis of cells of the pars recta only, and this necrosis was present only in the most proximal cells of this region. Cells very near the junction of the pars recta and thin loop of Henle appeared to escape the full extent of toxic insult suffered by cells of the early portion of the pars recta. This could result if the toxin were almost completely taken up by epithelial cells, thereby removing the toxin from the urinary filtrate, prior to the junction of the pars recta and thin loop. At higher concentrations of HFP, necrosis extended all the way down to the thin loop, and at highest concentrations, also extended proximally to the pars convoluta, which would be consistent with this postulate.

Another proposal why cells of the pars recta appear more susceptible to necrosis is that structural and functional differences between cells of various segments of the proximal tubule render different

cells susceptible to varying degrees and types of injury. This may involve different or special types of metabolism, or different enzymes within different renal segments.

Necrosis of the pars recta has also been demonstrated following renal hypotension (32,37).. High levels of sodium chloride passing through the macula densa of the juxtaglomerular apparatus (as in a state of diuresis) has been shown to result in local triggering of the renin-angiotensin system resulting in decreased blood flow to the kidney (38). Therefore, one may speculate that damage induced by fluoroalkenes may result in activation of the renin-angiotensin system, via the macula densa, causing a state of sustained renal cortical hypoperfusion. Since cells of the pars recta lie in the outer stripe of the medulla, which is poorly vascularized (39), they may suffer ill effects of hypotension more severely than cells of the pars convoluta. In the present study, histology of kidneys from rats exposed to the lowest concentration of HFP revealed necrosis of the more proximal cells of the pars recta, but the most distal cells were spared. This is inconsistent with the ischemic injury hypothesis since the most distal cells lie deepest in the outer stripe of the medulla, and therefore should be the most likely candidates for necrosis.

CONCLUSION

CTFE and HFP have been shown to disrupt renal function and cause necrosis of proximal tubules of the kidneys of rats. Since there is potential for human exposure to these compounds, via accidental industrial exposure, care should be exercised when dealing with possible sources of exposure to them. A human study of renal function in acute or chronically exposed workers has not been done.

If one assumes the maximum allowable levels of human exposure to be one tenth the level shown to be threshold for toxicity in animals, then this study would suggest that human exposure be restricted to less than 10 ppm CTFE and probably less than 30 ppm HFP for a four hour time period. It must be emphasized however that this standard applies only to acute exposure, and not long term chronic exposures, to which industrial workers may be subjected.

The findings of this study emphasize the necessity for studies of chronic low level exposures to CTFE and HFP, in order to evaluate any long term irreversible pulmonary effects and carcinogenic potential, as well as irreversible renal effects which may occur. This in turn may provide information crucial to establishment of proper hygienic guidelines for human exposure and regulations for safe use and handling of these compounds in the work place.

REFERENCES

1. Krespan, C. G. Organic fluorine chemistry. *Science* 150:13 (1965).
2. Clayton, J. W. Toxicology of the fluoroalkenes: Review and Research Needs. *Environ. Health Perspect.* 21:255 (1977).
3. Ferstandig, L. L. Warning: toxic fluoroorganic compound. *Chem. Eng. News* 44(49):6,8 (1966).
4. Dilley, J. W., Carter, V. L., and Harris, E. S. Fluorine ion excretion by male rats after inhalation of one of several fluoroethylenes or hexafluoropropene. *Toxicol. Appl. Pharmacol.* 27:582 (1974).
5. Danishevskii, S. L. and Kochanov, M. M. On the toxicology of some fluoroorganic compounds. *Gig. Tr. Prof. Zabol.* 5:3 (1961).
Cited in: Clayton, J. W. Toxicology of the fluoroalkenes: Review and Research Needs. *Environ. Health Perspect.* 21:255 (1977).
6. Waritz, R. S. and Kwon, B. K. The inhalation toxicity of pyrolysis products of polytetrafluoroethylene heated below 500 degrees centigrade. *Am. Indust. Hyg. Assoc. J.* 29:19 (1968).
7. Brubaker, R. E. Pulmonary problems associated with the use of polytetrafluoroethylene. *J. Occup. Med.* 19(10):693 (Oct. 1977).
8. Cook, E. W. and Pierce, J. S. Toxicology of fluoro-olefins. *Nature* 242:337 (1973).
9. Raventos, J. and Lemon, P. G. The impurities of fluothane: their biological properties. *Brit. J. Anesthesia* 37:716 (1965).
10. Clayton, J. W. Fluorocarbon toxicity and biologic action. *Fluorine Chem. Rev.* 1:197 (1967).
11. Lucia, H. L., Burton, A. K., Anderson, R. C., Stock, M. F., and Alarie, Y. C. Renal damage in mice following exposure to pyrolysis products of polytetrafluoroethylene. *J. Combust. Toxicol.* 5:270 (Aug. 1978).
12. Rosalki, S. B. and Wilkinson, J. H. Urinary lactic dehydrogenase in renal disease. *Lancet* 2:327 (1959).
13. Crockson, R. A. Lactate dehydrogenase in renal disease. *Lancet* 1:140 (1961).

14. Plummer, D. T. and Leathwood, P. D. Some properties of lactate dehydrogenase found in human urine. *Biochem. J.* 103:172 (1967).
15. Bhargava, A. R., Khater, A. R., and Gunzel, P. The correlation between lactate dehydrogenase activity in urine and serum and experimental renal damage in the rat. *Toxicol. Letters* 1:319 (1978).
16. Breedis, C., Florey, C. M., and Furth, J. Alkaline phosphatase level in relation to renal injury. *Arch. Path.* 36:402 (1943).
17. Plummer, D. T. and Ngaha, E. O. Urinary enzymes as an index of kidney damage by toxic compounds. In Nephrotoxicity, (ed.) Fillastre, J. P. (1977).
18. Stroo, W. E. and Hook, J. B. Enzymes of renal origin as indicators of nephrotoxicity. *Toxicol. Appl. Pharmacol.* 39:423 (1977).
19. Gomori, G. Microtechnical demonstration of phosphatase in tissue sections. *Proc. Soc. Exper. Biol. and Med.* 42:23 (1939).
20. Kabat, E. A. and Furth, J. Histochemical study of distribution of alkaline phosphatase in various normal and neoplastic tissues. *Am. J. Path.* 17:303 (1941).
21. Gomori, G. and Furth, J. Distribution of phosphatase in normal organs and tissues. *J. Cell. and Comp. Physiol.* 17:71 (1941).
22. Evan, A. P. and Dail, W. G. The effects of sodium chromate on the proximal tubules of the rat kidney: fine structural damage and lysozymuria. *Lab. Invest.* 30:704 (1974).
23. Ganote, G. E., Reimer, K. A., and Jennings, R. B. Acute mercuric chloride nephrotoxicity: an electron microscopic and metabolic study. *Lab. Invest.* 31:633 (1975).
24. Andrews, P. M. Scanning electron microscopy of the nephritic kidney. *Virchows Arch. B.* 17:195 (1975).
25. Mazze, R. I., Cousins, M. J., and Kosek, J. C. Strain differences in metabolism and susceptibility to the nephrotoxic effects of methoxyflurane in rats. *J. Pharmacol. Exp. Ther.* 184:481 (1973).
26. Barthelmai, W. and Czok, R. Enzymatische Bestimmungen der Glucose in Blut, Liquor und Harn. *Lin. Wochenschr* 40:585 (1962).

27. Folin, O. and Wu, H. A system of blood analysis. J. Biol. Chem. 38:81 (1919).
28. Skeggs, L. T. An automatic method for colorimetric analysis. Amer. J. Clin. Path. 28:311 (1957).
29. Marsh, W. H. and Fingerhut, B. Amer. J. Clin. Path. 28:681 (1957).
30. Handler, P. The effects of various inhibitors of carbohydrate metabolism in vivo. J. Biol. Chem. 161:53 (1945).
31. Wilfong, R. F. and Neville, D. M. The isolation of a brush border membrane fraction from rat kidney. J. Biol. Chem. 245:6106 (1970).
32. Kriesberg, J. I., Bulger, R. E., Trump, B. F., and Nagle, R. B. Effects of transient hypotension on the structure and function of rat kidney. Virchows Arch. (B. Cell Pathol.) 22:121 (1976).
33. Cousins, M. J., Mazze, R. I., Kosek, J. C., Hitt, B. A., and Love, F. A. The etiology of methoxyflurane nephrotoxicity. J. Pharmacol. Exp. Ther. 190:530 (1974).
34. Stonard, M. D. Further studies on the site and mechanism of action of S-(1,2 dichlorovinyl)-L-cysteine and S-(1,2 dichlorovinyl)-3-mercaptopropionic acid in rat liver. Bioch. Pharmacol. 22:1329 (1973).
35. Gandolfi, A. J., Associate Professor, University of Arizona, personal communication, August, 1979.
36. Roesinger, B., Schiller, A., and Taugner, R. A freeze-fracture study of tight junctions in the pars convoluta and pars recta of the renal proximal tubule. Cell Tiss. Res. 186:121 (1978).
37. Thurau, K., Dahlheim, H., Gruner, A., Mason, J., and Granger, P. Activation of renin in the single juxtaglomerular apparatus by sodium chloride in the tubular fluid at the macula densa. Circulation Res. 31/32 Suppl. II:182 (1972).
38. Dobyan, D. C., Nagle, R. B., and Bulger, R. E. Acute tubular necrosis in the rat kidney following sustained hypotension: physiologic and morphologic observations. Lab. Invest. 37(4): 411 (1977).
39. Moffatt, D. B. and Fourman, J. The vascular pattern of the rat kidney. J. Anat. 97:543 (1963).