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Chlorotrifluoroethylene Nephrotoxicity in Rats: A Subacute Study

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ABSTRACT

Chlorotrifluoroethylene Nephrotoxicity in Rats: A Subacute Study. Buckley, L.A., Clayton, J.W., Nagle, R.B. and Gandolfi, A.J. (1982). *Fundam. Appl. Toxicol.* 2:181-186. Male Fischer-344 rats were exposed via inhalation to a sublethal concentration (395 ppm ± 33 ppm; 1882 mg/m^3) of the nephrotoxin chlorotrifluoroethylene (CTFE) for 4 h per day for 5 consecutive days. Within 1 day after the first exposure, rats exhibited diuresis, increased water intake, decreased urine osmolality, increased urinary lactic dehydrogenase activity and increased plasma creatinine and urea nitrogen. When animals were exposed repeatedly, values for these parameters declined or returned to control levels during the exposure sequence in a manner comparable to rats receiving the single exposure. By the third day post exposure, coagulative necrosis involving primarily the pars recta, but extending to the pars convoluta, of the proximal tubule was present. Regeneration was apparent by the third day of exposure, and additional necrosis was minimal despite further exposures. Daily levels of urinary inorganic fluoride, an index of CTFE metabolism, were increased to 3-6 μmoles/24 h/rat during the exposure sequence which coincided with a brief elevation in serum fluoride at the end of each exposure. Adaptation to CTFE is evident either through changes in the metabolism or disposition of CTFE or from a refractive property of the regenerating tissue to CTFE.

INTRODUCTION

Chlorotrifluoroethylene (CTFE) is a gaseous fluoroalkene used in the production of fluoropolymers. In rats, the primary toxicity elicited at low to moderate concentrations of CTFE is nephrotoxicity (Clayton, 1977). Recently, Potter *et al.* (1981) have described several functional and morphological changes in the kidneys of male Fischer-344 rats after 4-h acute inhalation exposures to CTFE (100-540 ppm). In those studies, regeneration of the renal proximal tubules was reported within four days post exposure. In long-term inhalation studies with rats exposed repeatedly to 15-150 ppm of CTFE, rats developed degenerative changes in the renal tubules (Clayton, 1977).

Evaluation of the nephrotoxic effects elicited by repeated exposure to CTFE is important because, as an industrial chemical, there is a potential for chronic human exposure. The objective of the present study was to characterize, in greater detail, the renal toxicity of CTFE in rats after repeated exposure to CTFE, with special emphasis on the time course of the injury and recovery in the kidney.

METHODS

Animals

Male Fischer-344 rats (Microbiological Assocs., Walkersfield, MD) weighing from 150-200 g were randomly paired and housed in stainless steel metabolism cages. Animals were allowed at least five days to adapt to their surroundings and had free access to food (Wayne Lab Blox) and tap water.

Chemicals

Compressed CTFE and certified standards of CTFE in air were obtained from Matheson (Cucamonga, CA). Purity was determined by gas chromatography/mass spectometry as previously described (Potter *et al.*, 1981). The CTFE was 99% pure and the certified CTFE standards were 95% pure. Impurities were not conclusively identified but were thought to be highly fluorinated analogues of the parent compound. All other chemicals and biochemicals used were of reagent grade or better.

TABLE 1
Kidney Weight/Body Weight Ratios of Rats Following Repeated 4-h Exposures to 395 ppm CTFE[A]

Number of Days of Exposure	$\frac{\text{Kidney Weight}^B}{\text{Body Weight}} \times 10^3$[C]	
5	9.2 ± 1.0	(8)[D]
4	10.8 ± 1.5	(4)[D]
3	8.6 ± 0.6	(6)[D]
2	7.9 ± 0.6	(4)[D]
1	8.1 ± 0.9	(6)[D]
Control	6.8 ± 0.5	(12)

[A]All animals sacrificed 1 day after last exposure.
[B]Both kidneys.
[C]Values are means plus or minus standard deviations and numbers in parentheses indicate number of animals per group.
[D]Significantly different from control at the 0.05% confidence level or below.

This study represents work done in partial fulfillment of the Master of Science degree in Toxicology by L.A.B.

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Exposure system

The 180 L exposure chamber was constructed from Lucite plastic and supplied with an overhead fan for mixing and circulation. Chamber temperature was maintained at 25 ° ± 2 °C. Four galvanized steel cages, which could contain 6 rats each without overcrowding or condensation problems, were placed inside the chamber. The air flow rate was approximately 8 L/min. CTFE was delivered directly in the airflow stream via a double syringe infusion pump (Harvard Apparatus, Millis, MA). The concentration of the fluoroalkene was analyzed every half hour by gas chromatography (Potter *et al.*, 1981). Quantification was by peak height comparison of the gas chromatogram with that derived from the certified CTFE standards.

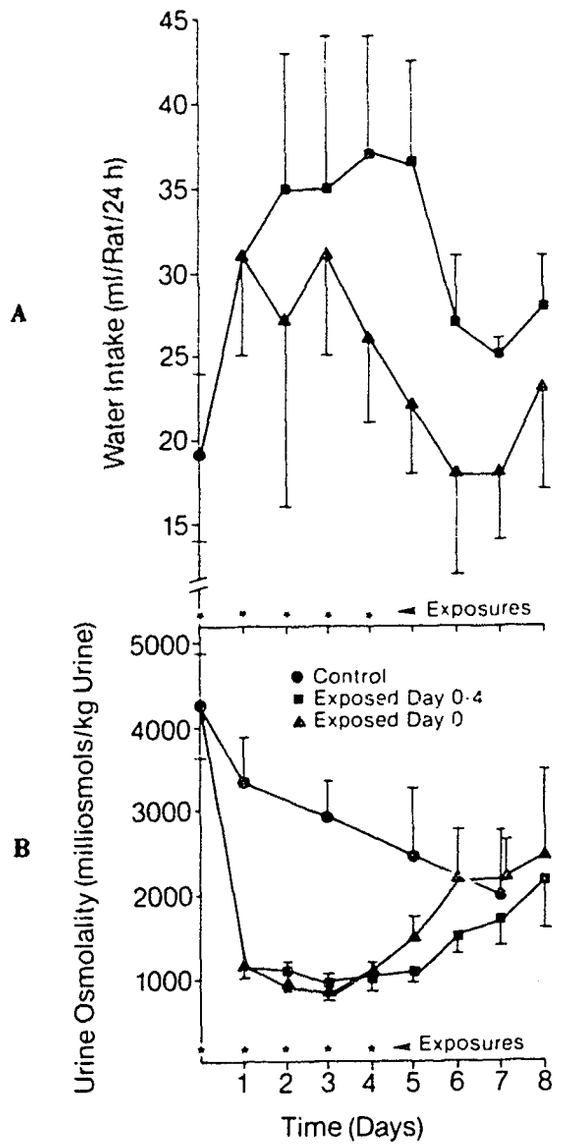

FIG. 1A. Daily water intake volumes of rats following single and repeated 4-h exposures to 395 ppm CTFE. (N=33 initially, diminishing to a minimum of N=4 or 6 after serial sacrifices; control N=111).

FIG. 1B. Daily urine osmolalities following single and repeated 4-h exposures of rats to 395 ppm CTFE. (N=36 initially, diminishing to a minimum of N=4 or 6 after serial sacrifices; control N=41, likewise diminishing).

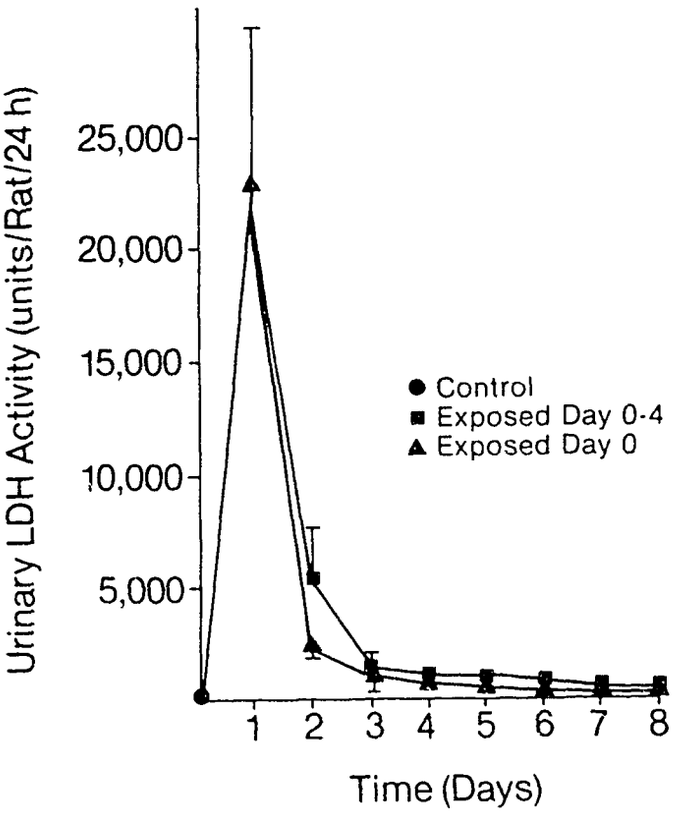

FIG. 2. Daily levels of urinary LDH excreted by rats following single and repeated 4-h exposures to 395 ppm CTFE. (N=35 initially, diminishing to a minimum of N=4 or 6 after serial sacrifices; control = 213 ± 261 units/24h/rat, N=104).

Exposures

Animals were exposed to 395 ± 33 ppm (1882 mg/m^3) CTFE for 4 h per day for 5 consecutive days or for a single exposure. After each 4-h exposure, rats were returned to their metabolism cages for urine collection and observation. Control animals were handled in the same manner except that they were not exposed to CTFE and control data was generated by pooling control animal data and pre-exposure data. Approximately 8 animals were sacrificed 24 h following 1, 2, 3, 4 or 5 exposures. In a group designated for serum fluoride measurements, rats were sacrificed 0, 2, or 20 h after 1, 2, 3, or 5 exposures to CTFE.

Urine analyses

Twenty-four-hour urine samples were collected from pairs of rats in plastic cups containing mineral oil to retard evaporation and 5 μg streptomycin and 20 μg gentamicin to prevent bacterial growth. Pre-exposure samples were obtained in the 24-h period before the initial exposure.

Urine osmolality was measured by vapor pressure osmometry (Wescor Vapor Pressure Osmometer, Logan, UT). Levels of urinary inorganic fluoride were determined by the method described by Fry and Taves (1970) using a fluoride specific ion electrode. Urinary lactic dehydrogenase activities (LDH) were analyzed using dialyzed urine as previously described (Potter *et al.*, 1981).

Blood analyses

Animals were killed by cervical dislocation and blood was obtained with a heparinized syringe from the inferior vena cava. Samples were certrifuged and the plasma analyzed for urea nitrogen and creatinine on a Technicon AutoAnalyzer. Serum inorganic fluoride was determined at 0, 2, and 20 h postexposure using the method described for urinary fluoride measurement.

Histology

At the time of sacrifice, kidneys from half the animals were directly fixed in 10% buffered formalin. The remaining kidneys were perfused *in vivo* with 2% glutaraldehyde in 0.1 M phosphate buffered saline, pH 7.2 (Griffith *et al.*, 1967). After perfusion, one kidney was cut longitudinally, the other, sagittally, and they were stored in refrigerated 10% buffered formalin. All kidneys were dehydrated in graded alcohols, embedded in paraffin, and stained with periodic acid Schiff stain (PAS) for light microscopic examination.

Statistics

Tests of significance of difference between two means were calculated using Students's t-test. Probability of 0.05 inferred a significant change from controls.

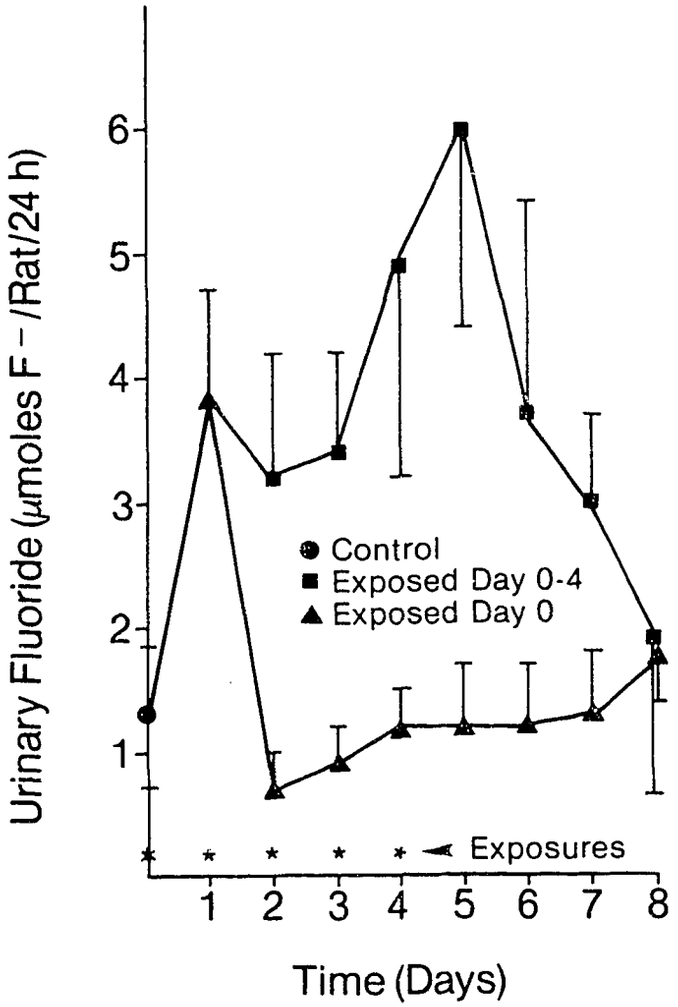

FIG. 3. Total urinary inorganic fluoride excreted by rats 24 h following single and repeated 4-h exposures to 395 ppm CTFE. (N=35 initially, diminishing to a minimum of N=4 or 6 after serial sacrifices; control N=102).

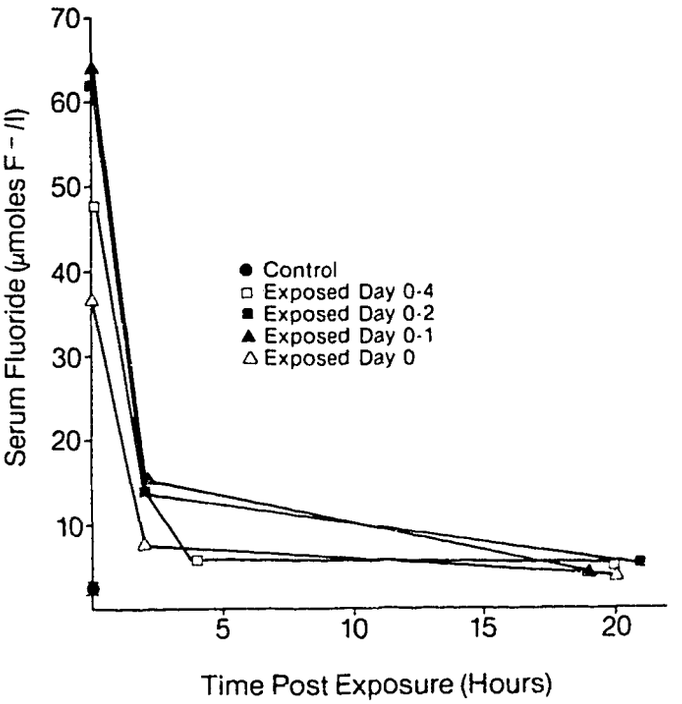

FIG. 4. Serum concentrations of inorganic fluoride in rats following single and repeated 4-h exposures to 395 ppm CTFE. (N=2; N=3 for control).

RESULTS

General health

The general appearance and behavior of the rats was unremarkable during the experimental period. However, a few CTFE-exposed animals had flecks of what appeared to be dried blood around the mouth and nares, which might be due to the respiratory tract irritation known to be elicited by CTFE (Clayton, 1977). Upon sacrifice, all exposed animals, whether singly exposed or repeatedly exposed, had increased kidney weight/body weight ratios (Table 1).

Urine osmolality and water intake

After a single exposure to 395 ppm CTFE, rats exhibited increased water intake (Figure 1A) and depressed urinary osmolalities (Figure 1B) which returned to normal by day 7. These parameters showed the same trend for animals exposed repeatedly to CTFE, though it took slightly longer to return to control levels. Control values for urine osmolality are expressed individually, rather than one pooled valued, because a slight decrease was noted over time.

Urinary LDH activity

An acute and dramatic increase in urinary LDH activities was observed after the first exposure. Despite repeated exposure, LDH activity normalized rapidly (Figure 2).

Inorganic fluoride

The daily levels of inorganic fluoride in the urine of rats exposed once or repeatedly to CTFE were monitored in order to provide a crude index of the degree of metabolism of CTFE. Rats exposed once demonstrated an acute elevation in urinary inorganic fluoride which was normalized by the second day post-exposure. In animals repeatedly exposed, urinary inorganic fluoride levels were increased throughout the exposure

TABLE 2
Serum Creatinine and BUN Levels in Rats Exposed Once or Five Times to 4-h Exposures of 395 ppm CTFE[A]

Number of Exposures	Serum Creatinine[B] (mg%)	BUN[B] (mg%)
1	1.2 ± 0.2 (12)[C]	45 ± 7 (4)[C]
5	0.5 ± 0.0 (3)	22 ± 4 (6)
Control	0.4 ± 0.1 (11)	19 ± 2 (4)

[A]All animals sacrificed 24 hours post-exposure.

[B]Values are means plus or minus standard deviations, and numbers in parentheses indicate number of animals per group.

[C]Significantly different from control at the 0.05% confidence level or below

sequence, especially from Days 4 and 5, and showed a somewhat delayed return to baseline levels by Day 8 (Figure 3).

Because inorganic fluoride is itself a classic renal toxin, serum levels were also measured. Serum inorganic fluoride levels were immediately elevated (35-65 μmoles) and then sharply reduced by 2 h post-exposure (Figure 4). Control levels were realized by 20 h post-exposure and before a subsequent re-exposure, if one was scheduled.

Blood analyses

Both plasma creatinine and urea nitrogen were elevated after the first exposure and returned to control levels by 24 h after the fifth exposure (Table 2).

Histopathologic examination

Light microscopic examinations of kidney sections revealed focal coagulative necrosis which was localized primarily in the pars recta of the proximal tubule in rats exposed to CTFE. With repeated exposures to CTFE, a retrograde extention along the proximal tubule involving the pars convoluta and outer cortical region became increasingly apparent. The maximum degree of necrosis was observed after the third day of exposure (Figure 5).

Tubular regeneration was characterized by the appearance of flattened epithelial cells with basophilic cytoplasm lining widely dilated tubules. These cells have little or no brush border in their early stages of regeneration. Mitotic figures were present. This regeneration, readily apparent 24 h after the third exposure, was quite advanced by the end of the exposure sequence (Figure 6).

DISCUSSION

The acute nephrotoxicity of inhaled CTFE in male Fischer-344 rats has been previously characterized (Clayton, 1977; Potter *et al.*, 1981). This study was designed to characterize the subacute nephrotoxicity of CTFE in rats to provide information relevant to the potential low dose exposure of humans to CTFE and other compounds behaving like CTFE.

Measurements of urinary osmolalities and water intake volumes showed similar results in rats exposed once or rats exposed repeatedly suggesting a deficiency in tubule concentrating ability of exposed animals. The elevated levels of serum creatinine and BUN after a single exposure show that plasma clearance ability may be acutely affected, as has been previously noted (Potter, 1981). After 5 exposures, however, both parameters were normalized. Thus, these commonly used indicators of renal injury would not be useful unless performed at the beginning of an exposure sequence. Similarly, repeated doses of mercuric chloride administered to rats orally did not affect levels of serum creatinine or BUN (Kluwe, 1981).

Measurement of urinary LDH activity provided a convenient and sensitive indication of acute proximal tubular necrosis, as has been observed by other investigators (Potter *et al.*, 1981; Bhargava *et al.*, 1978; Tandon *et al.*, 1980; Plummer and Ngaha, 1977; Plummer *et al.*, 1979). The 100-fold increase in activity which occurred after the initial 4-hour exposure was striking. With repeated exposures, the levels returned towards control despite continued exposure. Several others have noted this trend of initially high enzyme levels in the urine of animals treated with known nephrotoxins, and the subsequent decreased levels which remain low despite repeated exposure (Wachsmuth and Wirz, 1979; Kluwe, 1981). Wachsmuth and Wirz (1979) have shown good correlation between LDH activity in the urine and proximal tubular necrosis in rats receiving 8-10 daily injections of cephaloridine. In our study, however, it is debatable as to whether the degree of necrosis observed after 3 to 5 exposures correlated with the only slightly elevated LDH activity. By the third or fourth exposure, necrosis does extend further into subcoritcal and cortical regions. The amount of LDH in these areas (P1 and P2 segments, corresponding to subcortical and cortical pars convoluta of the proximal tubule) as determined histochemically by Jacobsen (1969), indicates that one could expect a notable increase in LDH excretion with injury to these segments.

Urinary inorganic fluoride determination showed that metabolism of CTFE, at least to the extent of defluorination, continued throughout the exposure sequence. Levels of inorganic fluoride in the urines of exposed animals seemed to increase after the third exposure (possibly indicating improved renal function), and it took 2-3 days for them to return to control. The possibility of accumulation and sequestration of inorganic fluoride, or of a compound undergoing defluorination, must be considered.

In his studies of the acute nephrotoxicity of CTFE and hexafluoropropene (HFP), Potter suggested that inorganic fluoride was not the etiologic agent of concern (Potter *et al.*, 1981). This study further supports his findings in that serum fluoride levels measured after exposure(s) were moderately elevated for a short time only, and were sharply decreased by 2 h post-exposure. Studies of the renal effects of methoxyflurane and enflurane, in which inorganic fluoride was considered the agent responsible for diuresis, showed high levels of serum fluoride which were maintained for up to 60 h post-anesthesia (Barr *et al.*, 1974).

The similar trends in measurements of renal function or renal injury for rats exposed once or repeatedly to CTFE suggest tolerance. Furthermore, histopathologic examination revealed that additional necrosis sustained after the third day of exposure was minimal and superimposed on an extensive regeneration process.

The mechanism of resistance to repeated insult may involve a number of factors. Balazs (1974) described the evolution of tissue resistance characterized by development of a specific lesion and then a specific resistance (of limited duration and apparently related to turnover rate of target cells). Others have seen this phenomenon after repeated dosing with cephaloridine (Wachsmuth and Wirz, 1979), gentamicin (Gilbert *et al.*, 1979), mercuric chloride (Prescott and Ansari, 1969), and mercuric chloride after administration of other nephrotoxic compounds (Tandon *et al.*, 1980). Certainly the injury is a specific one, and after destruction of the susceptible area, it

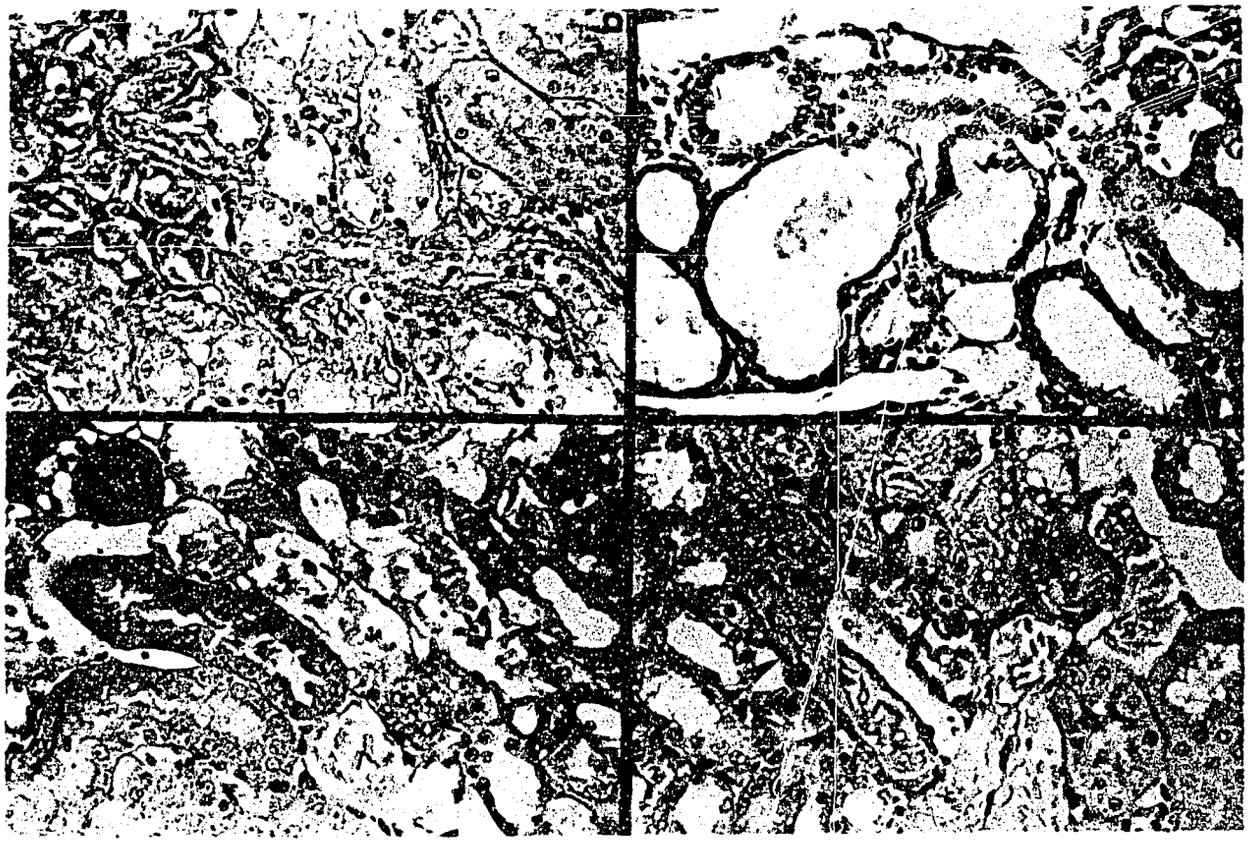

FIG. 6. Light micrographs of rat kidneys following sacrifice 24 h after exposure(s) to 395 ppm CTFE. (a) Following one exposure, note necrotic pars recta proximal tubules; uninjured tubules may also be seen; (b) after two exposures, arrows indicate the earliest signs of regeneration; (c) after three exposures, a more advanced stage of regeneration may be seen, as shown by arrows; and (d) following five exposures, very advanced regeneration is apparent. PAS × 600.

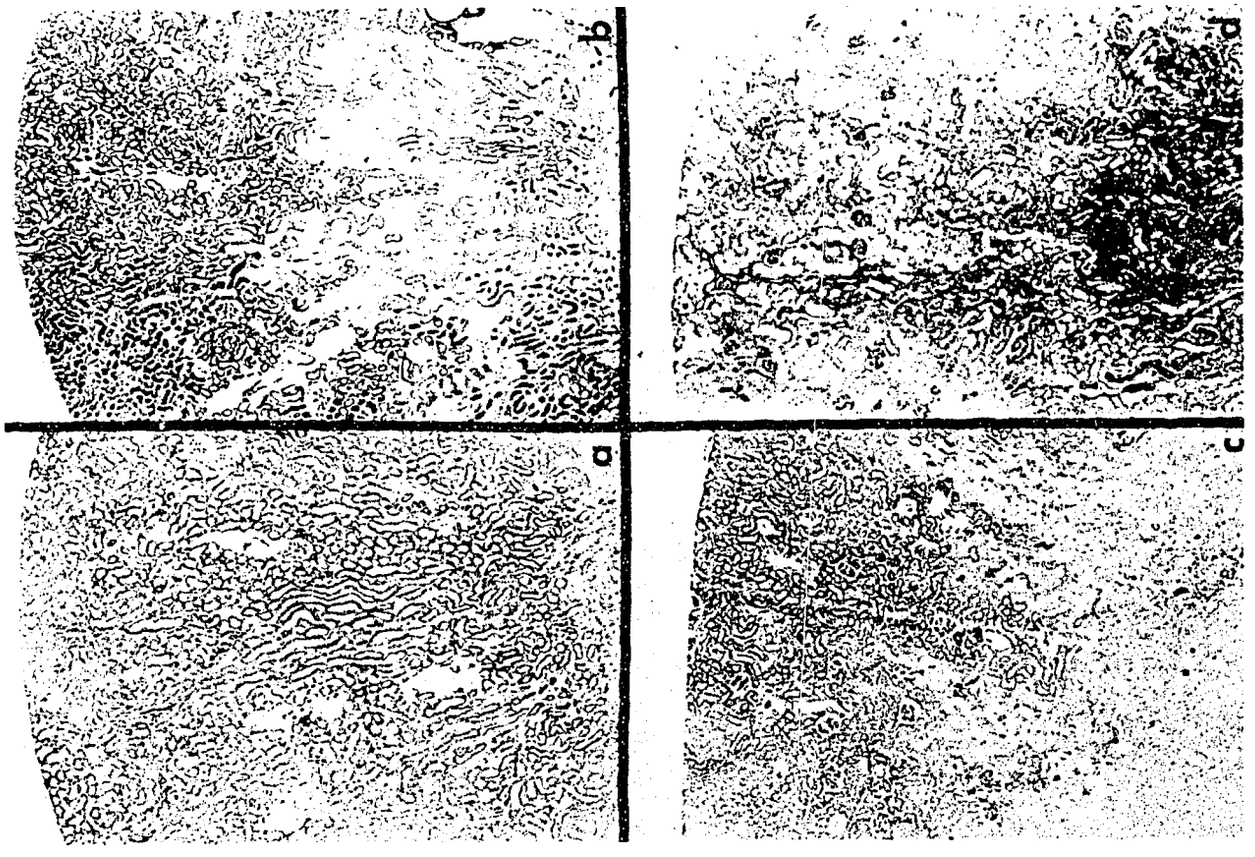

FIG. 5. Light micrographs of rat kidneys following 1, 2, 3, or 5 4-h exposures (a-d respectively) to 395 ppm CTFE. (a) control; (b) necrotic tubules are confined to the pars recta; (c) damage involving the proximal pars recta and extending upwards along the medullary rays; (d) necrosis fully extended into the outer cortex. PAS × 60.

may be that the newly regenerated, less differentiated cells do not exhibit the vulnerability of the mature epithelial cells, be it due to an altered physiologic or morphologic property of the cell (*i.e.* a change in permeability properties) or an altered metabolism of the toxin. With regards to metabolism, investigators have reported the nephrotoxicity of bioactivated halogenated vinyl cysteine conjugates derived from CTFE which are injurious specifically to the pars recta of the proximal tubule, similar to that injury observed in this study after inhalation of CTFE (Gandolfi *et al.*, 1981; Hassall *et al.*, 1981). Also, Bonhaus and Gandolfi (1981) have shown CTFE to react *in vitro* with glutathione in a microsomal system (liver or kidney). Their study presents evidence that CTFE may be bioactivated to a vinyl cysteine conjugate via cytosolic transferases and reaction with glutathione, the conjugate being subsequently activated by a C-S lyase to the toxic agent. The high activity of C-S lyase found in the renal brush border may account for the specific nephrotoxicity observed.

In summary, this work indicates that short-term repeated 4-h exposures of male rats to 395 ppm CTFE elicits nephrotoxicity with morphologic changes reflecting injury to the proximal tubule and changes in renal function. Recovery is rapid, and tolerance is observed as evidenced by similar changes in renal function of animals exposed once or for five days. Also, there is little increase in the severity of necrosis in animals exposed repeatedly after what appears to be a maximum damage incurred after the 3rd day of exposure.

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REFERENCES

Balazs, T. (1974). Development of tissue resistance to toxic effects of chemicals. *Toxicol.* 2:247.

Barr, G.A., Cousins, M.J., Mazze, R.I., Hitt, B.A. and Kosek, J.C. (1974). A comparison of the renal effects and metabolism of enflurane and methoxyflurane in Fischer 344 rats. *J. Pharmacol. Exp. Ther.* 188(1):257.

Bhargava, A.R., Khater, A.R. and Gunzel, P. (1978). The correlation between lactate dehydrogenase activity in urine and serum and experimental renal damage in the rat. *Toxicol. Letters* 1:319.

Bonhaus, D. and Gandolfi, A.J. (1981). Conjugation and bioactivation of chlorotrifluoroethylene. *Life Sciences* 29:2399.

Clayton, J.W. (1977). Toxicology of the fluoroalkenes: Review and research needs. *Environ. Health Perspect.* 21:255.

Gandolfi, A.J., Nagle, R.B., Soltis, J.J. and Plescia, F. (1981). Nephrotoxicity of halogenated vinyl cysteine compounds. *Research Commun. Chem. Path. Pharmacol.* 33:249.

Gilbert, D.N., Houghton, D.C., Bennett, W.M., Plamp, C.E., Reger, K. and Porter, G.A. (1979). Reversibility of gentamicin nephrotoxicity in rats: recovery during continuous drug administration. *Proc. Soc. Exp. Biol. Med.* 160:99.

Griffith, L.D., Bulger, R.E. and Trump, B.F. (1967). The ultrastructure of the functioning kidney. *Lab. Invest.* 16(2):220.

Hassall, C.D., Gandolfi, A.J. and Brendel, K. (1981). Depression of tubular active transport by halogenated hydrocarbons and their metabolites. *Pharmacologist* 23:191.

Jacobsen, N.O. (1969). The histochemical localization of lactic dehydrogenase isoenzymes in the rat nephron by means of an improved polyvinyl alcohol method. *Histochemie* 20:250.

Kluwe, W.M. (1981). Renal function tests as indicators of kidney injury in subacute toxicity studies. *Toxicol. Appl. Pharmacol.* 57:414.

Plummer, D.T. and Ngaha, E.O. (1977). Urinary enzymes as an index of kidney damage by toxic compounds. In *Nephrotoxicity*, (ed.) Fillastre, J.P.

Plummer, D.T., Ngaha, E.O., Wright, P.J., Leathwood, P.D. and Blake, M.E. (1979). The sensitivity of urinary enzyme measurements for detecting renal injury. In *Current Problems in Clinical Biochemistry*:9, (ed.) Dubach, U.C. and Schmidt, U.

Potter, C.L., Gandolfi, A.J., Nagle, R.B. and Clayton, J.W. (1981). Effects of inhaled chlorotrifluoroethylene and hexafluoropropylene on the rat kidney. *Toxicol. Appl. Pharmacol.* 59(3):431.

Prescott, L.F. and Ansari, S. (1969). The effects of repeated administration of mercuric chloride on exfoliation of renal tubular cells and urinary glutamic-oxaloacetic transaminase activity in the rat. *Toxicol. Appl. Pharmacol.* 14:97.

Tandon, S.K., Magos, L. and Cabral, J.R.P. (1980). Protection against mercuric chloride by nephrotoxic agents which do not induce metallothionein. *Toxicol. Appl. Pharmacol.* 52:227.

Wachsmuth, E.D. and Wirz, H. (1979). Relevance of enzyme evaluations in 24h urine to rat kidney injury caused by i.v. cephaloridine injection. In *Current Problems in Clinical Biochemistry*:9, (ed.) Dubach, U.C. and Schmidt, U.