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Inhalation Toxicity of an Isomeric Mixture of Hydrochlorofluorocarbon (HCFC) 225 in Male Rats

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A blend of the hydrochlorofluorocarbon isomers HCFC-225ca and HCFC-225cb has been proposed as a potential substitute for CFC-113, an important solvent and cleaning agent. The toxicity following repeated inhalation of an HCFC-225 isomer mixture was assessed in male Crl:CDBR rats. Three groups of 10 male rats were exposed to the test compound in air at design concentrations of 500, 5000, and 13,000 ppm. Rats were exposed 6 hr/day, 5 days/week for 2 weeks. A control group of 10 male rats was exposed to air only. Decreased serum cholesterol, triglycerides, and glucose; dose-related increased mean absolute and relative liver weights; and microscopic hepatocellular hypertrophy were present at all exposure concentrations. Hepatocellular hypertrophy correlated ultrastructurally to proliferation of peroxisomes. Clinical chemical parameters and organ weight and morphologic changes in the liver were reversible following 14 days of recovery. © 1992 Society of Toxicology.

Chlorofluorocarbons (CFCs) are believed to be major contributors to the seasonal depletion of ozone over the Antarctic continent. The hydrochlorofluorocarbon compounds (HCFC), along with the hydrofluorocarbon compounds, have emerged as alternatives for some CFC market segments. A mixture of the HCFC-225 isomers, HCFC-225ca ($CF_3CF_2CHCl_2$) and HCFC-225cb ($CFHClCF_2CF_2Cl$), has been proposed as a potential substitute for CFC-113, an important solvent and cleaning agent (Manzer, 1990). It was necessary to assess the inhalation toxicity of this HCFC-225 isomer mixture since inhalation is the exposure route of industrial and environmental importance for this compound. The acute inhalation toxicity of this mixture was considered very low with a 4-hr approximate lethal concentration of 31,000 ppm in rats (Du Pont Co., 1990). On the basis of this data, design concentrations of 500, 5000, and 13,000 ppm were selected for a 2-week inhalation study. The purpose of this study was to determine the toxic effects and their reversibility following repeated inhalation of sublethal concentrations of an HCFC-225 isomer mixture in male rats.

MATERIALS AND METHODS

Compound. An approximately 50:50 isomeric mixture of HCFC-225ca (3,3-dichloro-1,1,1,2,2-pentafluoropropane) with HCFC-225cb (1,3-dichloro-1,1,2,2,3-pentafluoropropane) was provided by Du Pont Chemicals (E. I. du Pont de Nemours and Co., Wilmington, DE). The actual measured concentrations were 53.6% HCFC-225ca, 42.5% HCFC-225cb, 3.0% HCFC-225a, and 0.9% HCFC-225ba. The control group was exposed to air only in a 38-liter cylindrical, glass exposure chamber.

Animals and husbandry. Male, 7-week-old Crl:CDBR rats (Charles River Breeding Laboratories, Raleigh, NC) were used in this study. Rats have historically been used in safety evaluation studies, and their use was specified by the sponsor. This strain of rats has been well characterized in this laboratory.

Rats were exposed nose-only to varying concentrations of HCFC-225 isomer mixture in air (see below for details). Following exposures, rats were housed in pairs in 8 in. × 14 in. × 8 in. suspended, stainless steel wire-mesh cages. During the 2-week recovery period (i.e., after 10 exposures), rats were housed individually in the stainless steel wire-mesh cages. Except during exposures, Purina Certified Rodent Chow No. 5002 and water were available *ad libitum*.

Animal rooms were maintained on a timer-controlled, 12 hr/12 hr light/dark cycle. Environmental conditions of the rooms were targeted for a temperature of 23 ± 2°C and relative humidity of 50 ± 10%.

General experimental design. The effects of repeated inhalation of the HCFC-225 isomer mixture were assessed in four groups of 10 male rats. Three test groups were exposed nose-only to design concentrations of 500, 5000, or 13,000 ppm of the HCFC-225 isomer mixture in air. A control group matched for age, sex, and body weight was exposed simultaneously to air only. Following the end of daily 6-hr exposure periods, the rats were returned to their cages. Body weights and clinical, clinical pathological, and pathological parameters were assessed. Rats were exposed 6 hr/day, 5 days/week for 2 weeks.

At the end of the exposure period (test Day 12), blood and urine samples were collected from all rats for clinical pathological analyses. Five randomly selected rats per group were euthanized for pathological examination. After 14 days of recovery, the remaining five rats from each group were given the same examinations.

Atmosphere generation. Vapor atmospheres of the HCFC-225 isomer mixture were generated by metering the gaseous compound from a cylinder. Air was introduced into the cylinder and bubbled through the test material. This pushed the vapor of the HCFC-225 mixture through Teflon tubing and into a mixing flask. Dilution air added to the mixing flask at approximately 25 liters/minute carried the HCFC-225/air mixture into a 38-liter cylindrical, glass exposure chamber. Each chamber was fitted with a baffle and miniature fan to assist in even dispersion of the vapor. Desired atmospheric concentrations of the HCFC-225 isomer mixture were produced by regulating the flow of the vapor of the mixture from the cylinder. The exposure chamber atmosphere was exhausted through a Sethco charcoal filter prior to discharge

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into the fume hood. The control group was exposed to air only in a 38-liter cylindrical, glass exposure chamber.

Analysis of the test atmospheres. Chamber atmospheres for each exposure group were monitored approximately every 30 min. Duplicate samples of chamber atmosphere were automatically sampled and analyzed with a computer driven Hewlett–Packard Model 5880A gas chromatograph equipped with a flame ionization detector. Samples were chromatographed isothermally at 50°C on a 20 in. × $\frac{1}{8}$ in. o.d. stainless steel column packed with 2% OV-101 liquid phase on a 100/120 mesh WHP B1-B5 solid phase. Nitrogen was used as a carrier gas. The injection port and detector temperatures were maintained at 75 and 250°C, respectively. The atmospheric concentrations of the vapor of the HCFC-225 isomer mixture were determined using a computer to compare the detector response of samples with standard curves. Standards were prepared prior to exposures by quantitatively diluting liquid HCFC-225 isomer mixture in air.

During each exposure, chamber temperature were measured with a mercury thermometer, relative humidity was measured with a Belfort Model 566 psychrometer, and chamber oxygen content was measured with a Biosystems Model 3100R oxygen monitor.

Body weights and clinical observations. During the exposure period, all rats were weighed and observed for clinical signs of toxicity before each exposure. Observations for clinical signs were also made during and immediately following each exposure. During the 14-day recovery period, rats were weighed and observed daily, weekends excluded.

Clinical pathology measurements. Urine samples were collected overnight from all rats after the 9th exposure and from the remaining rats on the 13th day of recovery. Samples were analyzed for volume, osmolality, urobilinogen, pH, urine fluoride, hemoglobin or occult blood, glucose, protein, bilirubin, and ketones (Multistix, Ames Division, Miles Laboratory, Elkhart, IN). Urine fluoride was determined using an Orion Model 96-09-00 ion specific electrode. The color and transparency of each sample was noted, and the sediment from each sample was examined microscopically.

A blood sample was taken from the orbital sinus of each rat after the 10th exposure and from each remaining rat on the 14th day of recovery. Rats were lightly anesthetized with carbon dioxide prior to being bled. Blood samples were analyzed for erythrocyte count, hemoglobin concentration, hematocrit, platelet count, leukocyte count, and relative numbers of neutrophils, band neutrophils, lymphocytes, atypical lymphocytes, eosinophils, monocytes, and basophils. Mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration (Wintrobe erythrocyte indices) were calculated from the erythrocyte data.

Serum clinical chemistry parameters were measured on a Coulter DACOS clinical chemistry analyzer using Coulter DART reagents. Serum activities of alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase were determined. Serum concentrations were determined for the following: glucose, urea nitrogen, bilirubin, triglycerides, cholesterol, creatinine, total protein, albumin, globulin (calculated from the total protein and albumin concentrations), calcium, phosphorus, sodium, potassium, and chloride. In addition, plasma fluoride levels were determined using the same method as for urine fluoride measurement.

Pathology. The 10 rats per exposure group were each subdivided into groups of five based on computer generated random number tables. The first 5 rats per group were euthanized after the 10th exposure and were examined for gross and histopathologic alterations. All rats were euthanized by sodium pentobarbital anesthesia and exsanguination. On the 14th day of the postexposure period, the remaining rats of each group were similarly terminated and evaluated. The lungs, liver, kidneys, brain, and testes were weighed at necropsy. Representative samples of the following tissues were fixed in 10% neutral buffered formalin: heart, lungs, mesenteric lymph nodes, nose, larynx/pharynx, trachea, liver, pancreas, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, kidneys, urinary bladder, spleen, thymus, thyroid gland, adrenal glands, brain, spinal cord, and peripheral (sciatic) nerve. Eyes, bone marrow (sternal), testes, and epididymides were fixed in Bouin's fixative. Fixed tissues were processed routinely for paraffin embedment, sectioned at a nominal thickness of 5 μm, and stained with hematoxylin and eosin for microscopic examination.

Retrospectively, formalin-fixed sections of liver from selected 0-day recovery animals in all groups, and from one animal each in the control and the 13,000 ppm recovery groups, were postfixed in osmium tetroxide. These liver sections were processed routinely for electron microscopy and examined with a Zeiss EM 10 transmission electron microscope.

Statistical analyses. Mean body weights and body weight gains during the exposure and recovery periods were determined for controls and all test groups. Final body and organ weights were also determined. Values for test groups were compared to controls using a one-way analysis of variance (ANOVA). When the F test from the ANOVA was significant, the Dunnett's test was used to compare the control group with each exposure group. Significance was judged at the 0.05 probability level.

For clinical pathology data, an ANOVA and a Bartlett's test were calculated for each sampling time. When the F test from the ANOVA was significant, the Dunnett's test was used to compare the control group with each exposure group. Significance was judged at the 0.05 probability level. When the results of the Bartlett's test were significant ($p \leq 0.005$), the Kruskal–Wallis test was employed and the Mann–Whitney U test was used to compare means from the control group to each exposure group. Significance was judged at the 0.05 probability level.

TABLE 1
Mean Atmospheric Concentrations of the HCFC-225ca/HCFC-225cb Mixtures[a]

Group No.	Design concentration	Analyzed concentration (ppm)			
		Mean	SD	Range	n
I	Air control[b]	0	0	0	106
III	500	510	51	360–720	215
V	5,000	5,000	830	1900–8,000	215
VII	13,000	13,000	1700	9000–20,000	215

[a] Values represent the means, standard deviations (SD), ranges, and total number of samples (n) from all exposures.

[b] Air-exposed procedural control group.

RESULTS

Chamber atmosphere analysis. In the test chambers, temperature ranged from 21 to 27°C, relative humidity ranged from 41 to 62%, and oxygen concentration ranged from 20 to 21%. In the control chamber, temperature ranged from 22 to 25°C, relative humidity ranged from 45 to 65%, and chamber oxygen concentration was maintained at 21%. The mean chamber atmospheric concentrations of the vapor of the HCFC-225 isomer mixture vapor are presented in Table 1. In general, the measured exposure concentrations were in close agreement with the design concentrations. Thus, in the present report, exposures in each group were considered to be 500, 5000, and 13,000 ppm.

Clinical observations. All animals survived to the termination of the study and were euthanized by design. No compound-related clinical signs were observed during the exposure or recovery periods. No significant differences in mean body weights were observed among any of the exposure groups when compared to controls. Rats exposed to 5000 or

TABLE 2
Clinical Pathology Results for Male Rats Exposed to HCFC-225ca/HCFC-225cb

Test	Days on test	Group exposure concentration (ppm) 0	500	5000	13,000
Cholesterol (mg/dl)	12[a]	62 (11)[b]	43 (7)*	49 (10)*	45 (16)*
	26[c]	62 (7)	60 (7)	72 (17)	66 (10)
Triglycerides (mg/dl)	12	42 (22)	16 (11)*	29 (13)	34 (16)
	26		Test not performed		
Glucose (mg/dl)	12	151 (15)	141 (6)	138 (6)	136 (10)*
	26	157 (17)	165 (7)	167 (15)	172 (28)
Albumin (g/dl)	12	3.1 (0.1)	3.3 (0.2)*	3.3 (0.1)*	3.4 (0.1)*
	26	3.2 (0.2)	3.3 (0.2)	3.3 (0.3)	3.3 (0.1)
Globulin (g/dl)	12	3.0 (0.2)	2.4 (0.2)*	2.5 (0.3)*	2.5 (0.2)*
	26	3.3 (0.2)	3.0 (0.3)	3.3 (0.1)	3.4 (0.2)
Urine fluoride (μg/16 hr)	12	23.0 (2.6)	30.5 (5.7)*	41.0 (6.7)*	47.7 (5.0)*
	26	38.3 (6.1)	37.9 (4.3)	42.3 (5.0)	38.0 (5.0)

[a] Twelve days on test with exposure to HCFC-225ca/HCFC-225cb (6 hr/day) for 10 of the 12 days; $n = 5$ for all groups.
[b] Group mean and standard deviation.
[c] Twenty six days on test with 10 days of exposure (6 hr/day) to HCFC-225ca/HCFC-225cb during the first 12 days followed by 14 days of recovery (no exposure); $n = 5$ for all groups.
* Statistically different from control group mean, $\alpha = 0.05$.

13,000 ppm of the HCFC-225 isomer mixture had significantly decreased mean body weight gains during Days 9–10 and 11–12 of the exposure period.

Clinical pathology. Significant clinical pathology findings are presented in Table 2. Exposure-related decreases in mean serum cholesterol, triglyceride, and glucose concentrations were present in all exposure groups. Decreases in serum cholesterol were statistically significant at all exposure concentrations but were not dose related. Decreases in serum triglycerides were not dose related and were statistically significant in only the 500 ppm dose group. Decreases in serum glucose were statistically significant in rats exposed to 5000 and 13,000 ppm.

Dose related and statistically significant increases in urine fluoride concentration were present in all exposure groups. However, no statistically significant changes were present in serum fluoride concentrations in any exposure group.

Other clinical pathological findings included statistically significant increases in albumin concentration and decreases in globulin concentration in all exposure groups.

No biologically significant clinical pathology changes were observed in any exposure group following a 14-day recovery period. Triglyceride concentrations were inadvertently not measured following the recovery period.

TABLE 3
Mean Final Body and Liver Weights in Male Rats Exposed to HCFC-225ca/HCFC225cb

Concentration (ppm)	Mean final body weight (grams)	Mean absolute liver weight (grams)	Mean relative liver weight[a]
	12 Days on test[b]		
0	284.3 (8.6)	9.664 (0.882)	3.3965 (0.2458)
500	286.2 (11.9)	13.010 (0.905)*	4.5486 (0.3011)*
5,000	279.0 (12.8)	13.712 (2.398)*	4.9014 (0.7098)*
13,000	275.2 (8.5)	14.002 (1.232)*	5.0839 (0.3352)*
	26 Days on test[c]		
0	375.7 (27.5)	14.513 (1.672)	3.8550 (0.1789)
500	366.7 (16.8)	13.852 (1.673)	3.7708 (0.3369)
5,000	385.8 (22.2)	15.573 (1.444)	4.0445 (0.3978)
13,000	361.6 (10.9)	13.810 (1.368)	3.8175 (0.3339)

Note. Standard deviation is in parentheses.
[a] Percentage of body weight.
[b] Six hours per day for 10 of 12 days; $n = 5$ for all groups.
[c] Ten days of exposure during the first 12 days followed by 14 days of recovery (no exposure); $n = 5$ for all groups.
* Statistically different from control group mean, $\alpha = 0.05$.

Pathology. Following the final exposure, dose-related increases in mean absolute and relative (percentage of body weight) liver weights were present in all treated groups. These liver weight changes were reversible at all exposure concentrations following the 14-day recovery period (Table 3). No other statistically or biologically significant changes in organ weights were present in exposure groups relative to controls.

Microscopically, hepatocellular hypertrophy was present in the livers of rats in all treated groups following the final exposure. Hypertrophy was usually diffuse and affected hepatocytes had granular, eosinophilic cytoplasmic swelling and large, hypochromic nuclei (Figs. 1 and 2). Hypertrophy was

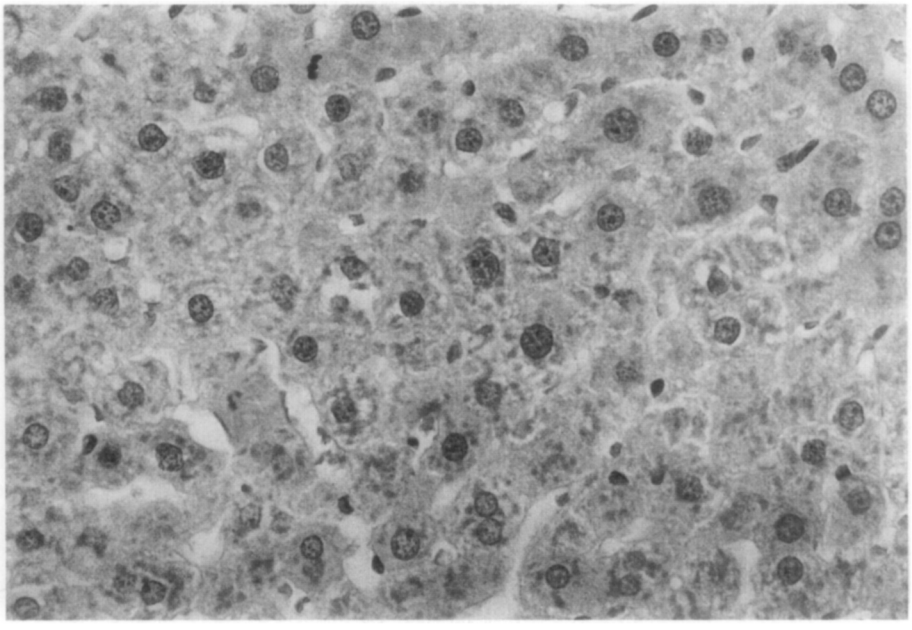

FIG. 1. Liver from a control rat. H&E ×425.

minimal to mild and was more conspicuous in the 13,000 ppm dose group compared to the 5000 or 500 ppm dose groups. Microscopic changes correlated ultrastructurally to increases in peroxisome numbers in the livers of rats in all exposure groups (Figs. 3 and 4). Profiles of smooth endoplasmic reticulum also appeared increased. Microscopic and ultrastructural changes in the liver were reversible following the 14-day recovery period. There were no other compound-related pathological changes in any of the tissues examined.

DISCUSSION

Exposure of male rats to the vapor of an isomeric mixture of HCFC-225ca and HCFC-225cb at design concentrations of 500, 5000 or 13,000 ppm produced increased liver weights and hepatocellular hypertrophy. This hypertrophy was associated with peroxisome proliferation. Clinical chemical findings, suggestive of alterations in lipid and glucose metabolism, were also present. Decreases in serum triglycerides

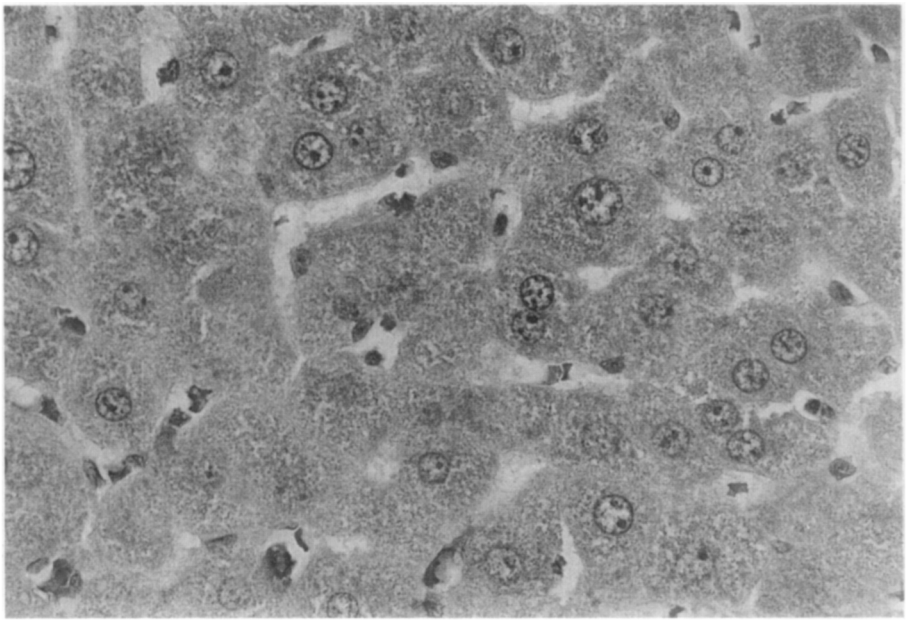

FIG. 2. Hypertrophy with increased cytoplasmic granularity of hepatocytes in the liver of a rat exposed to 13,000 ppm of HCFC-225 isomer mixture. H&E ×425.

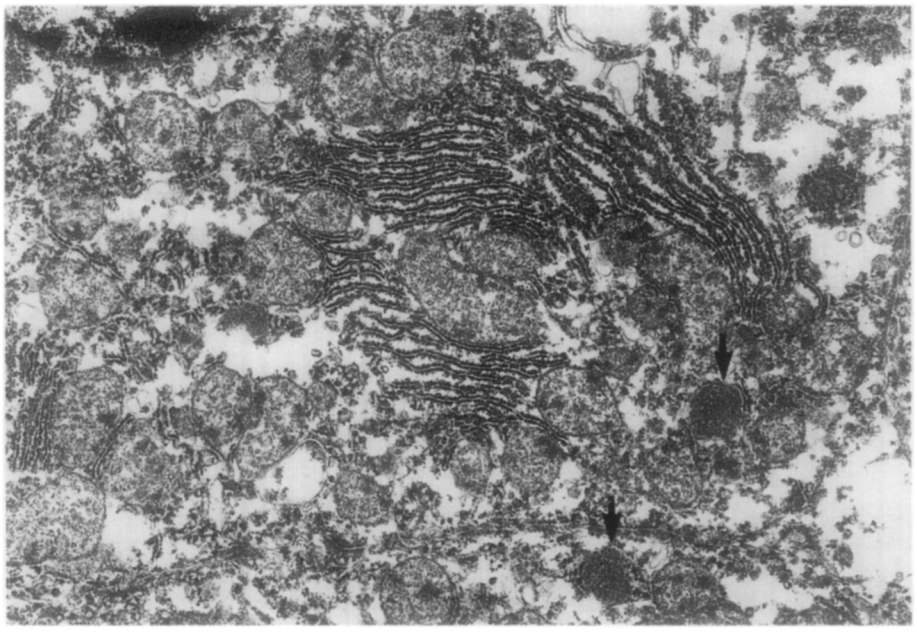

FIG. 3. Peroxisomes (arrows) in the liver from a control rat. ×13,000.

and cholesterol, though not dose related, may well represent treatment-related effects based upon similar findings following exposure to other fluoride-containing compounds (Malley *et al.*, 1990; Frame *et al.*, 1991). The biological significance of the changes in serum proteins is not known but may be related to alterations in liver metabolism. Increases in urinary fluoride concentrations were considered to be physiologically normal consequences of exposure to a fluoride-containing compound. Thus, these increases were not considered to be toxicologically significant effects.

All pathological and clinical pathological findings related to exposure to the HCFC-225 isomer mixture were reversible following a 14-day recovery period. This reversibility is consistent with the results of previous studies which demonstrated the rapid reversibility of the effects of most peroxisome proliferators following the termination of treatment

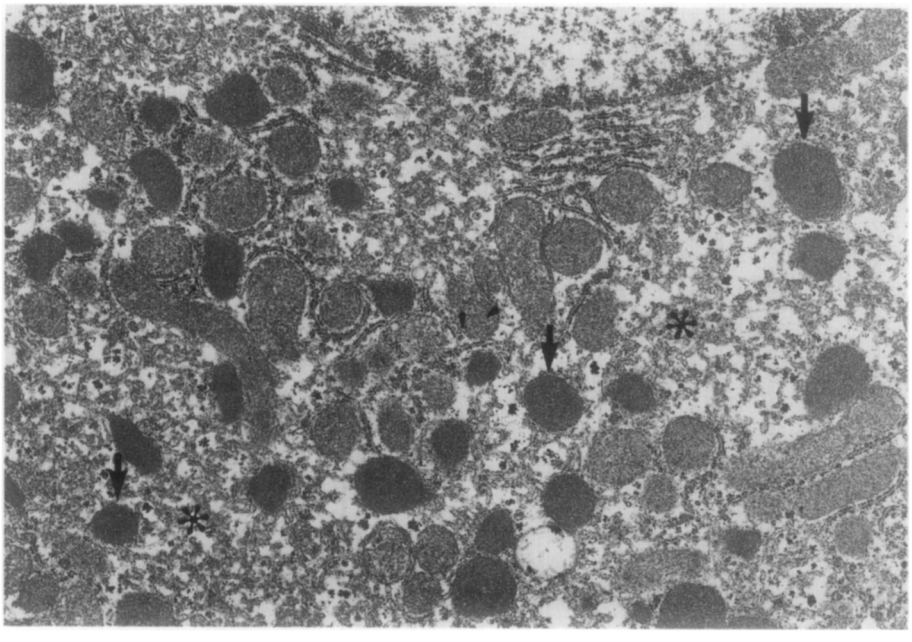

FIG. 4. Increased numbers of peroxisomes (arrows) and profiles of smooth endoplasmic reticulum (asterisks) in the liver of a rat exposed to 13,000 ppm of HCFC-225 isomer mixture. ×13,000.

(Stotts, 1988). Although triglyceride concentrations were inadvertently not measured after the recovery period, results of other studies on similar compounds have demonstrated that triglyceride concentrations in exposed rats are similar to controls following a 14-day recovery period.

Effects similar, though less potent, to those produced following exposure to the HCFC-225 isomer mixture were observed in a 90-day inhalation toxicity study in rats exposed to another hydrochlorofluorocarbon compound, HCFC-123 (Malley *et al.*, 1990). Changes included increases in liver weights, hypolipidemia, and hypoglycemia. In addition, hepatic peroxisomal β-oxidation activity, a marker for induction of hepatic peroxisomal proliferation, was increased in all treatment groups (Du Pont Co., 1989). Morphologic evidence of hepatocellular hypertrophy was not present in that study.

Hypolipidemia, hepatomegaly, and hepatic peroxisomal proliferation are produced by a number of compounds. In addition, hypolipidemia in association with hypoglycemia, which occurred following exposure to both the HCFC-225 isomer mixture and HCFC-123, has also been reported for the peroxisomal proliferators 2[5(4-chlorophenylpentyl)]-oxirane-2-carboxylate and valproic acid (Mannaerts and Veldhoven, 1988).

Pronounced species differences in chemically induced peroxisomal proliferation have been reported by a number of investigators. Male rats appear to be the species and sex most sensitive to chemically induced peroxisomal proliferation (Rodricks and Turnbull, 1987). Numerous *in vivo* and *in vitro* studies have demonstrated that compared to rodents, higher mammalian species, including humans, are considerably less sensitive or are insensitive to peroxisome proliferators (Stott, 1988). Thus, as previously noted (Rodericks and Turnbull, 1987; Stotts, 1987), species differences are important considerations in the interpretation of toxicology data, including oncogenicity data, from studies with peroxisome proliferators.

Two 28-day repeated dose inhalation toxicity studies were recently carried out on the individual isomers, HCFC-225ca and HCFC-225cb (Trochimowicz, 1991). In those studies, male and female rats were exposed to 60, 220, or 650 ppm of either HCFC-225ca or HCFC-225cb. Exposures to HCFC-225cb produced no toxic effects in rats at any exposure concentrations. However, in male rats, exposures to HCFC-225ca produced significant increases in absolute and relative liver weights at 220 and 650 ppm and microscopic hepatocellular hypertrophy at 650 ppm. All liver changes were reversible following a 2-week recovery period. No pathological changes were present in female rats at any of the exposure concentrations tested. Thus, male rats appeared to be more sensitive to the toxic effects of HCFC-225ca. The results of these studies suggest that the HCFC-225ca isomer is the component most responsible for the effects observed in the present study with an isomeric mixture of HCFC-225ca and HCFC-225cb.

In conclusion, inhalation exposure of male rats to an isomeric mixture of HCFC-225 produced organ weight and morphologic changes in the liver as well as clinical chemical findings of decreased serum cholesterol, triglyceride, and glucose concentrations.

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