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Effects of Short-term Oral Dosing of Polychlorotrifluoroethylene (PolyCTFE) on the Rhesus Monkey*

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Polychlorotrifluoroethylene (polyCTFE—primarily oligomers with 3–4 monomer units), a non-flammable hydraulic fluid for aircraft, was given daily for 15 days by oral gavage to four Rhesus monkeys at a concentration of 0.725 g kg^{-1}. The administered dose was at a level that had caused toxicity in rats. Steady-state blood and liver concentrations reached were the same in both species. In monkeys, polyCTFE did not cause the electrolyte, serum protein, liver enzyme and anemic disturbances previously seen in rats. Liver sections taken at 15 days, analyzed for palmitoyl Co-A beta-oxidation rates or by electron microscopy, showed no significant indication of peroxisomal proliferation. An increased blood urea nitrogen (BUN) at 15 days was the only clinical pathological abnormality seen in both monkeys and rats. Previously unobserved effects were increased triglycerides and glycogen depletion.

INTRODUCTION

Chlorotrifluoroethylene (CTFE, CAS no. 79-38-9) is a gaseous fluoroalkene used in the production of fluoropolymers. Polychlorotrifluoroethylene (polyCTFE, CAS no. 9002-83-9) is the base stock for a non-flammable hydraulic fluid for aircraft.[1] It contains CTFE oligomers blended to a viscosity of 3.1 cSt using primarily three (trimer) and four (tetramer) monomeric units. All future US Air Force hydraulic systems operating at or above 8000 psi must be able to use polyCTFE.[2]

In rats, the primary toxicity seen as a result of exposure to the vapor of monomer CTFE was nephrotoxicity.[3–6] Gad *et al.*[6] did see an increase in liver weights, but there were no other indicators that the liver is a target organ.

Rats exhibited increased water intake and decreased urinary osmolalities even when exposed to a single exposure of CTFE, suggesting histopathological changes in the renal tubules. Microscopic examination at necrospy revealed necrosis that was primarily localized in the pars recta of the proximal tubule.[4–6] There was, however, regeneration of the tubule epithelial cells by day 3 and full regeneration within 2 weeks.[4] Potter *et al.*[4] and Buckley *et al.*[5] found a dose-related increase in urinary lactate dehydrogenase (LDH), blood urea nitrogen (BUN) and serum creatinine at all inhalation exposure levels above 100 ppm, suggesting that the plasma clearance ability is affected. All these parameters returned to normal rapidly, even in animals receiving multiple exposures.

Inorganic fluoride, a probable metabolite of CTFE and a known renal toxin, was measured both as an indicator of metabolism and of toxic effect. There was a dose-related increase in inorganic fluoride in blood and urine, but again levels returned to baseline, although more slowly in repeated exposure experiments than in single exposure experiments.[4,5]

Clayton[3] conducted repeated exposure experiments that suggest a species-specific toxicity. In guinea pigs exposed to 300 ppm for 6 h per day, 5 days per week, less than 18 exposures produced death. In a study using dogs involving stepped exposures from 15 to 150 ppm, exposures of 50 ppm resulted in hematological changes, such as leukopenia, erythrocytosis, granulocytopenia and elevated plasma cholesterol. When the exposures were increased to 150 ppm, neurological disturbances were observed in the dog. Rabbits exposed to 250 ppm for 6 h per day for up to 110 days showed hypochronic anemia, a reduction of hemoglobin concentrations, a reduction in red blood cell count and leukopenia.

Sex might also be a factor in the toxicity of CTFE. A dose–response effect was evident in both sexes, with males being more sensitive.[6] A dose–response effect was most noticeable in causing an increase in both the absolute and relative liver weights in males. Although females were affected, males had a dose–response effect, significant differences in weight gain, urine creatinine and urine fluoride, and histopathological changes.

* The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, DHHS, National Institute of Health Publication 86-23, 1985, and the Animal Welfare Act of 1966, as amended. A portion of this study was conducted under DoD contract number F33615-85-C-0532.

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Efforts to understand CTFE toxicity and the fact that it is reversible following cessation of exposure has focused on CTFE metabolism. Studies of the metabolism of CTFE[7] indicated that it might be metabolized in the liver, in the presence of glutathione, to yield ultimately a CTFE analog of vinyl cysteine. In the kidney, the vinyl cysteine conjugate is metabolized to a sulfur-containing metabolite and pyruvate. When this analog was administered to animals, the renal proximal tubules showed the same toxic response as seen in animals that inhaled CTFE. Dohn and coworkers,[8,10] using both microsomal hepatic fractions and isolated renal tubules, presented data that showed that the nephrotoxicity is due to the enzymatic formation of the glutathione conjugate, which is metabolized by the sequential action of renal gamma-glutamyltransferase, cysteinyl-glycine dipeptidase and cysteine conjugate-lyase.

Acute toxicity studies of polyCTFE indicated that it has a low degree of toxicity.[11] There were no deaths among rabbits exposed dermally to a single dose of 3.7 g polyCTFE kg^{-1} body weight.[11] The oral LD_{50} in rats was >9.2 g kg^{-1}. PolyCTFE also showed no toxicity as a skin irritant, minimal toxicity as an eye irritant and a mild potential for skin sensitization.[11] Only 1 in 15 rats died after exposure for a 4 h period to atmospheres containing saturated-vapor concentrations of polyCTFE (4.7 mg l^{-1} or 234 ppm), although absorption was evidenced by elevated plasma and urine fluoride levels.[11] No target organ was identified by a single exposure to polyCTFE liquid or vapor.

However, a subchronic, 90-day, repeated-dose inhalation study of polyCTFE revealed an unexpectedly high degree of liver toxicity in the rat.[12] Groups of 10 male and 10 female rats were exposed to airborne concentrations of polyCTFE of 0.25, 0.50 or 1.0 mg l^{-1} for 6 h per day, 5 days per week for 13 weeks. All groups of rats gained weight throughout the exposure, but the weight gain was reduced markedly in rats exposed to the highest concentrations. At the end of the exposure, plasma alkaline phosphatase was elevated in all exposed rats, while plasma AST and ALT values were elevated only in the high-dose group. Liver weight increased for both male and female rats with increasing dose of polyCTFE. On post-mortem examination, the liver was enlarged greatly and showed a variety of changes in its cellular structures. Transmission electron microscopy revealed an increase in smooth endoplasmic reticulum, mitochondrial swelling and significant peroxisomal proliferation.[12]

The difference in toxicity between acute and subchronic studies suggests that the toxicity of polyCTFE may be due to metabolites that accumulate only after repeated dosing and cause peroxisome proliferation. Although the structural requirements for chemicals causing peroxisome proliferation are not understood fully, a structural moiety common to most peroxisome proliferators is a carboxylic acid or a functional group that is converted to a carboxylic acid.[13] The likely metabolic route is cytochrome P-450 oxidation of the polyCTFE, which results in an unstable intermediate and, finally, polyCTFE acid. The ability of a structurally similar compound, perfluoro-*n*-decanoic acid, to induce peroxisome proliferation in the rat[14] is noteworthy in this regard.

The significance of the observed peroxisome proliferation lies in its association with carcinogenicity in rodents, which may or may not be species-specific.[15–17] Reddy *et al.*[18] hypothesized that persistent proliferation of peroxisomes serves as an endogenous initiator of neoplastic transformation of hepatocytes by increasing the intracellular production of hydrogen peroxide by the peroxisomal oxidases. The implications of this potential mechanism to humans is debated. Studies of two chemicals, DEHP and Wy-14,643, indicate that peroxisome induction and carcinogenicity are not linked quantitatively, as the present theory suggests.[19] Peroxisome proliferation and the resulting liver carcinogenicity may be unique species-specific responses restricted to rats and mice, with questionable predictive value for humans.[15,16,20]

Griffith and Long[21] found the major sites of toxicity of ammonium perfluorooctanoate, a compound resembling polyCTFE acid, in the Rhesus monkey to be the gastrointestinal tract and the reticuloendothelial system, as opposed to the hepatotoxicity (peroxisome proliferation) seen in rats.

As a class, hypolipidemic agents cause proliferation of hepatic peroxisomes in rodents.[15] Svoboda *et al.*[22] did not observe hepatic peroxisomal proliferation in several non-rodent species given a hypolipidemic drug. Reddy *et al.*,[18] on the other hand, were able to induce peroxisome proliferation in primates with a hypolipidemic drug by increasing the dose.

The purpose of this investigation was to see if the changes observed in rats are present in non-human primates exposed to polyCTFE.

EXPERIMENTAL

Animals

Male Rhesus monkeys (*Macaca mulatta*) weighing 8–10 kg, fed Purina Certified Monkey Chow 5048 *ad libitum*, were used in the study.

Chemicals

Polychlorotrifluoroethylene (polyCTFE) was supplied by WRDC/MLBT (Non-Structural Materials Branch, Non-Metallic Materials Division, Materials Laboratory, AF Wright Research & Development Center, Wright-Patterson AFB, OH 45433). WRDC/MLBT received polyCTFE as halocarbon 3.1 oil from Halocarbon Products Corporation, North Augusta Industrial Park, PO Box 6369, North Augusta, SC 29841).

Exposures

Four Rhesus monkeys were dosed daily for 15 days with 0.725 g kg^{-1} polyCTFE through a nasogastric tube following anesthesia with ketamine·HCl. Four control animals received a comparable amount of water following anesthesia with ketamine·HCl.

Clinical observations

Animals were observed carefully by a veterinarian every day. Food consumption was monitored by the

animal care staff. Monkeys were weighed daily prior to each dose. Weights of 24 h fecal samples were obtained at 0, 7 and 15 days of dosing.

PolyCTFE concentrations in biological samples

Blood was collected for polyCTFE analysis and clinical pathology evaluations from a distal posterior leg or femoral vein on days 0, 7 and 15. Blood for polyCTFE analysis (0.1 ml) was transferred into 1.8 ml serum vials containing 1.0 ml of hexane. To reduce polyCTFE vaporization, the transfer occurred below the surface. PolyCTFE extraction was accomplished using an Evapotec® mixer overnight.

Tissues collected for polyCTFE analysis were weighed and then maintained in hexane on ice until homogenization. The tissues were homogenized using a Tekmar Tissue-mizer®, then mixed overnight on Haake Buchler Evapotec® mixer. Following centrifugation, the hexane layer was stored at −70°C but allowed to return to room temperature before analysis.

Tissue extracts were diluted with hexane to reduce the polyCTFE in the samples to <500 ng ml^{-1} for analytical purposes.

A Varian® 3700 gas chromatograph (GC) equipped with an electron capture detector (ECD) was used to analyze the biological samples. A Nelson® integration system was programmed to handle the GC output. PolyCTFE–hexane solution standards were used to quantitate the ECD signal. The usable standard range was 1–500 ng ml^{-1}.

The assumption of a theoretical 100% extraction efficiency for polyCTFE in blood and tissue was based on studies conducted in this laboratory.[11,12] A hexane solution of known concentration of polyCTFE was allowed to be mixed intimately with biological samples for sufficient time to permit uptake of the polyCTFE by tissues. The hexane solutions were analyzed by gas chromatography (the same system used for biological samples) to determine loss of polyCTFE to the tissues. There were no significant losses of polyCTFE to the tissues.

Clinical pathology

Blood collected on days 0, 7 and 15 was analyzed to determine clinical chemistry and hematology parameters. Clinical chemistry values were measured on a Kodak Ektachem 700XR clinical chemistry analyzer. Hematology values were measured on a Coulter S-Plus II cell counter.

Ultrastructural analyses

A laparotomy was performed on each animal at the end of the dosing period in order to obtain a liver wedge for analysis. A 1 mm slice of the liver wedge from each animal was collected for transmission electron microscopic examination. The liver slices were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and minced into 1-mm^3 pieces. The minced tissue was post-fixed with 2% osmium and was processed into plastic capsules (EPOX 812, Ernest F. Fullam, Inc., Latham, NY). Sections 1 μm thick were cut in order to identify centrolobular zones. Thin sections were cut from the centrolobular and intermediate zones of liver lobules. Thin sections stained with uranyl acetate and lead citrate were examined with a transmission electron microscope (Model 100B, Jeol USA, Inc., Peabody, MA) at 60 kV. For each animal, 20–30 photographs were taken of three or more hepatocytes that were representative of the liver for that animal. The number of peroxisomes were counted per 30,000 magnification visual field using an 8 × 10 in. photograph. An average of nine photographs were counted for each animal.

Palmitoyl-CoA oxidation

Peroxisomal proliferation was assessed by measuring the rate of palmitoyl-CoA oxidation in fresh liver homogenates (1:4, w/v, in 0.25 M sucrose), as described by Gray *et al.*[23]

Statistical analyses

Statistical analysis of peroxisome counts was conducted using the Student's *t*-test at the $P < 0.05$ level of significance. Other data obtained from treated animals were compared with those from control animals after 0, 7 and 15 days of dosing using a multi-factoral analysis of variance.

RESULTS

Clinical observations

Control animals consumed their daily allowance of food while monkey dosed with polyCTFE consistently did not. Despite the observed reduced food intake in treated monkeys, no statisticaly significant changes in animal or fecal weights were observed (Table 1).

PolyCTFE concentrations in blood and liver

Repeated daily oral dosing of Rhesus monkeys at 0.725 g kg^{-1} resulted in venous concentrations of CTFE trimer and tetramer reaching 2.0 and 1.8 mg l^{-1}, respectively, by 1 day after dosing. At 2 weeks, liver concentrations of trimer and tetramer were 70 and 100 mg l^{-1}, respectively.

Clinical chemistry

A significant increase above the control levels of triglycerides was evident at both days 7 (135%) and 15 (285%). A significant increase above the control value of BUN (36%) was evident at day 15 (Table 2).

Hematology

No significant changes in hematology parameters were evident.

Urinalysis

A significantly higher volume per hour (vol, h^{-1}) of urine in the treated group was evident prior to treatment (Table 3).

Table 1. Clinical observations[a]

	Animal weight (kg)		Fecal weight (g per 24 h)	
Day	Control	Treated	Control	Treated
0	9.6 ± 0.6	9.1 ± 1.3	93 ± 13	51 ± 28
7	8.8 ± 0.9	8.9 ± 1.0	70 ± 29	61 ± 44
15	8.7 ± 0.9	8.6 ± 1.3	67 ± 21	50 ± 34

[a] Values represent the mean ± the standard deviation of the mean. None of the treated data were significantly different from the control data.

Ultrastructural analysis

Repeated exposure of primates to polyCTFE for 15 days resulted in only minor changes in the hepatocytes. Mild to moderate mitochondrial swelling was seen in both the control (Fig. 1) and the exposed (Fig. 2) animals; this was probably a result of ischemia owing to the method of obtaining the biopsy (C. C. Dougherty, MD, personal communication). There was no evidence of peroxisomal proliferation (Figs 3 and 4). The difference between the mean number of peroxisomes for control primate liver, 2.5 ± 0.33 (±SEM), and exposed primate liver, 3.3 ± 0.42, was not statistically significant. A decrease in glycogen was seen in primates exposed to polyCTFE (Fig. 2 or Fig. 4).

Fatty acid beta-oxidation

At day 15 of the study, fatty acid beta-oxidation activity in livers was 3.0 ± 0.8 μM min^{-1} g^{-1} for control animals and 4.1 ± 2.8 μM min^{-1} g^{-1} for treated animals. The difference between control and treated animals was not statistically significant.

DISCUSSION

A difference in the toxic effects of polyCTFE between monkeys and rats was found, based on comparable dose and steady-state liver and blood concentrations. Monkeys dosed orally daily for 15 days with 0.725 g

Table 2. Clinical chemistry results[a]

Test	Day	Control	Treated	Test	Day	Control	Treated
BUN	0	18 ± 4	17 ± 3	CREA	0	1.3 ± 0.4	1.2 ± 0.2
	7	16 ± 4	17 ± 3		7	1.3 ± 0.3	1.0 ± 0.2
	15	14 ± 3	19 ± 4*		15	1.2 ± 0.3	1.1 ± 0.1
BUN/CR	0	14 ± 2.4	15 ± 0.9	GLU	0	74 ± 14	53 ± 12
	7	13 ± 4.6	17 ± 5.1		7	78 ± 7	75 ± 8
	15	12 ± 2.0	18 ± 4.2		15	72 ± 6	65 ± 9
CHOL	0	148 ± 21	113 ± 30	TRIG	0	49 ± 28	44 ± 23
	7	141 ± 20	119 ± 39		7	79 ± 30	186 ± 58*
	15	131 ± 21	116 ± 37		15	68 ± 31	262 ± 131*
AST	0	66 ± 33	41 ± 9	ALT	0	45 ± 22	41 ± 12
	7	55 ± 15	57 ± 18		7	55 ± 25	63 ± 29
	15	61 ± 30	83 ± 31		15	86 ± 88	118 ± 62
ALKP	0	145 ± 43	127 ± 43	GGT	0	58 ± 15	94 ± 51
	7	135 ± 58	131 ± 20		7	55 ± 13	104 ± 48
	15	144 ± 46	160 ± 45		15	60 ± 22	110 ± 46

[a] Values represent the mean ± the standard deviation of the mean. Asterisked values were significantly different ($P < 0.05$) from control data. Abbreviations and units used: BUN, blood urea nitrogen (mg dl^{-1}); CREA, creatinine (mg dl^{-1}); BUN/CR, BUN-to-creatinine ratio; GLU, glucose (mg dl^{-1}); CHOL, cholesterol (mg dl^{-1}); TRIG, triglycerides (mg dl^{-1}); AST, aspartate aminotransferase (IU l^{-1}); ALT, alanine aminotransferase (IU l^{-1}); ALKP, alkaline phosphatase (IU l^{-1}); GGT, gamma-glutamyl transferase (IU l^{-1}).

Table 3. Urinalysis results[a]

Test	Day	Control	Treated	Test	Day	Control	Treated
VOL/HR	0	21 ± 16	37 ± 3*	CR CLR/KG	0	2.0 ± 1.6	2.1 ± 0.9
	7	24 ± 11	26 ± 15		7	2.3 ± 0.9	2.5 ± 0.7
	15	21 ± 7	24 ± 16		15	2.5 ± 0.4	2.2 ± 1.3

[a] Values represent the mean ± the standard deviation of the mean. The asterisked value was significantly different ($P < 0.05$) from control data, even though treatment had not yet begun. Abbreviations and units used: VOL/HR, volume of urine per hour (ml l^{-1}); CR CLR/KG, ratio of creatinine clearance to animal weight (ml min^{-1} kg^{-1}).

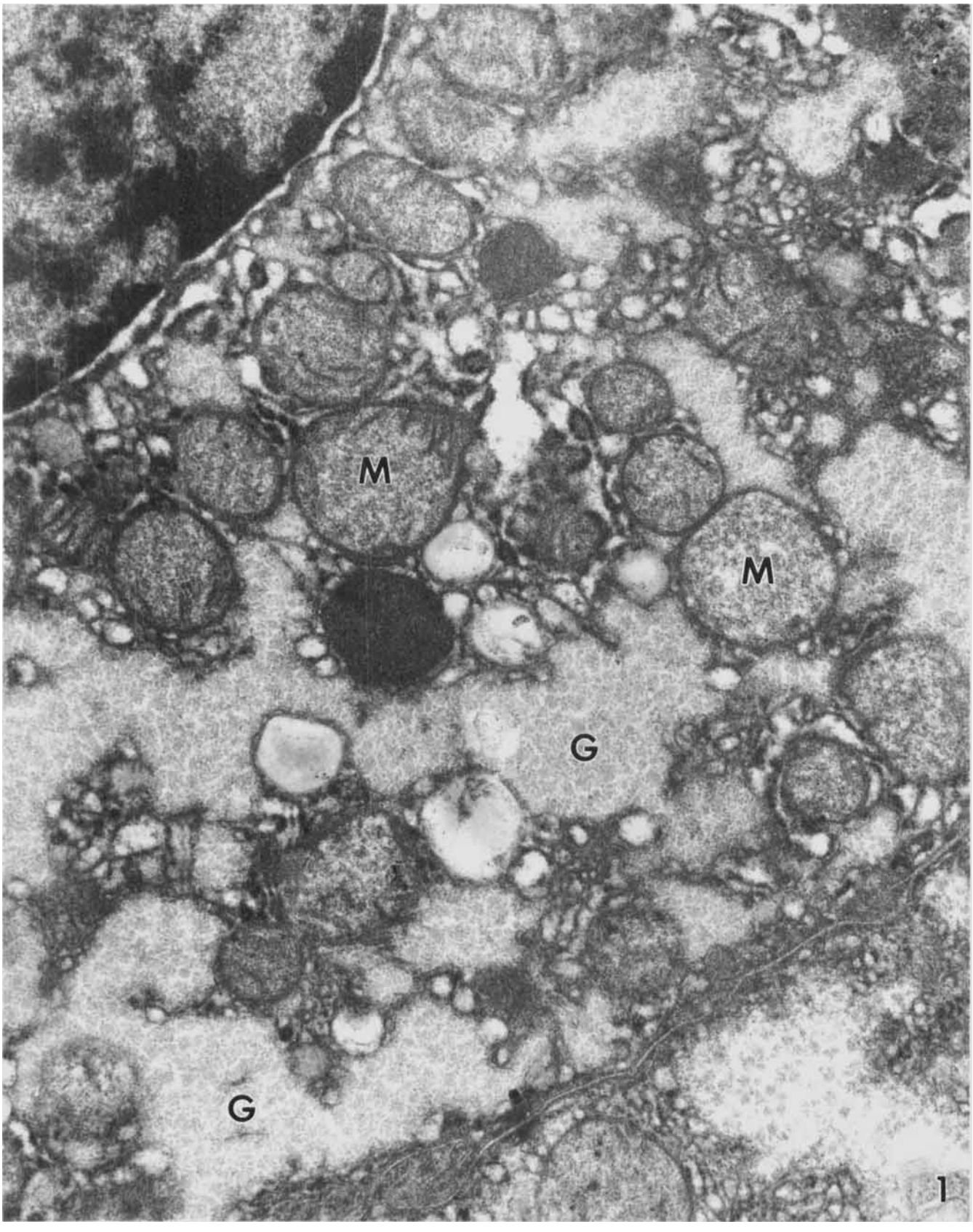

Figure 1. Control primate hepatocytes with glycogen (G) and swollen mitochondria (M). 30 000×.

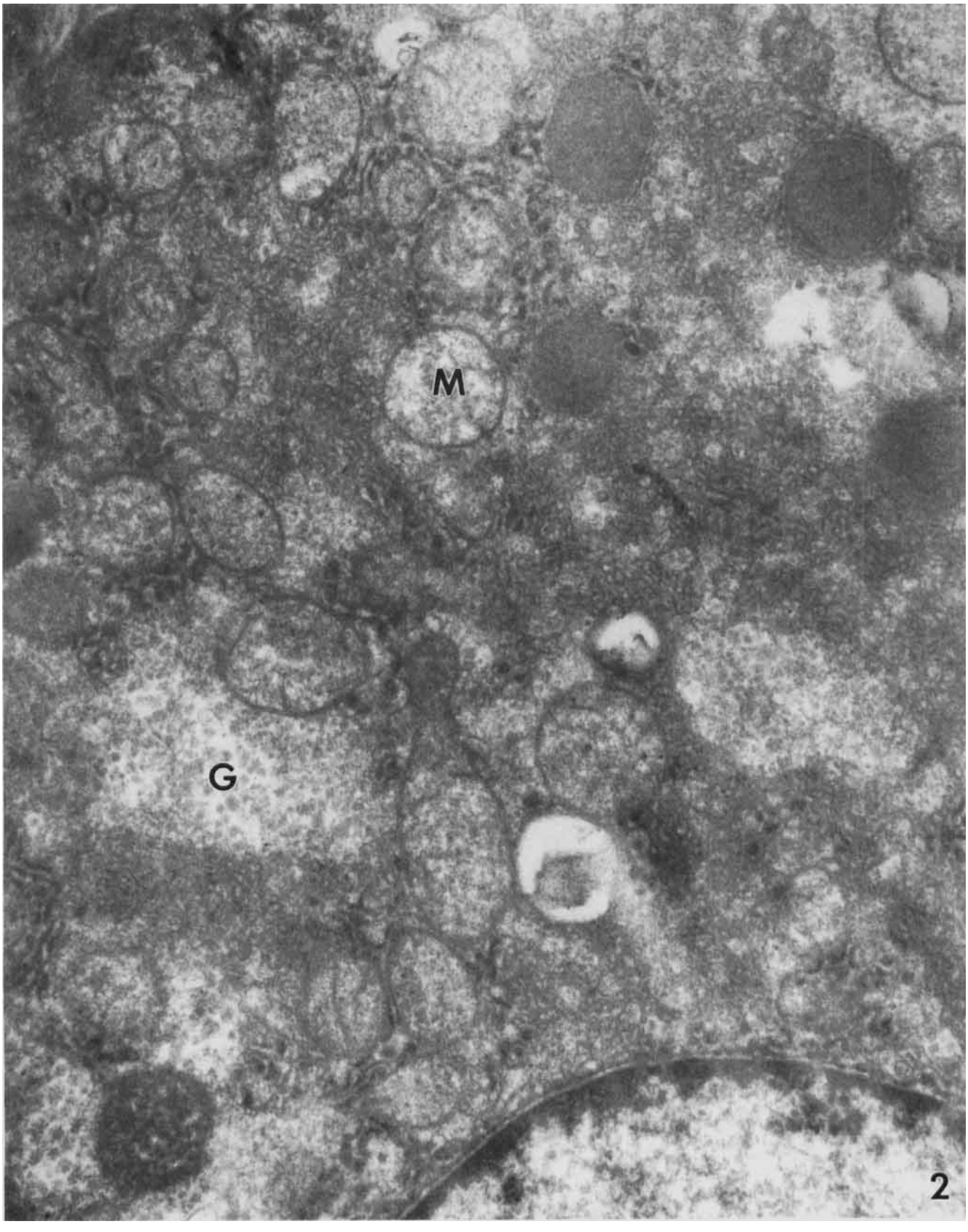

Figure 2. Primate hepatocyte from liver exposed to polyCTFE for 15 days, with swollen mitochondria (M) and showing a decreased amount of glycogen (G). 30 000×.

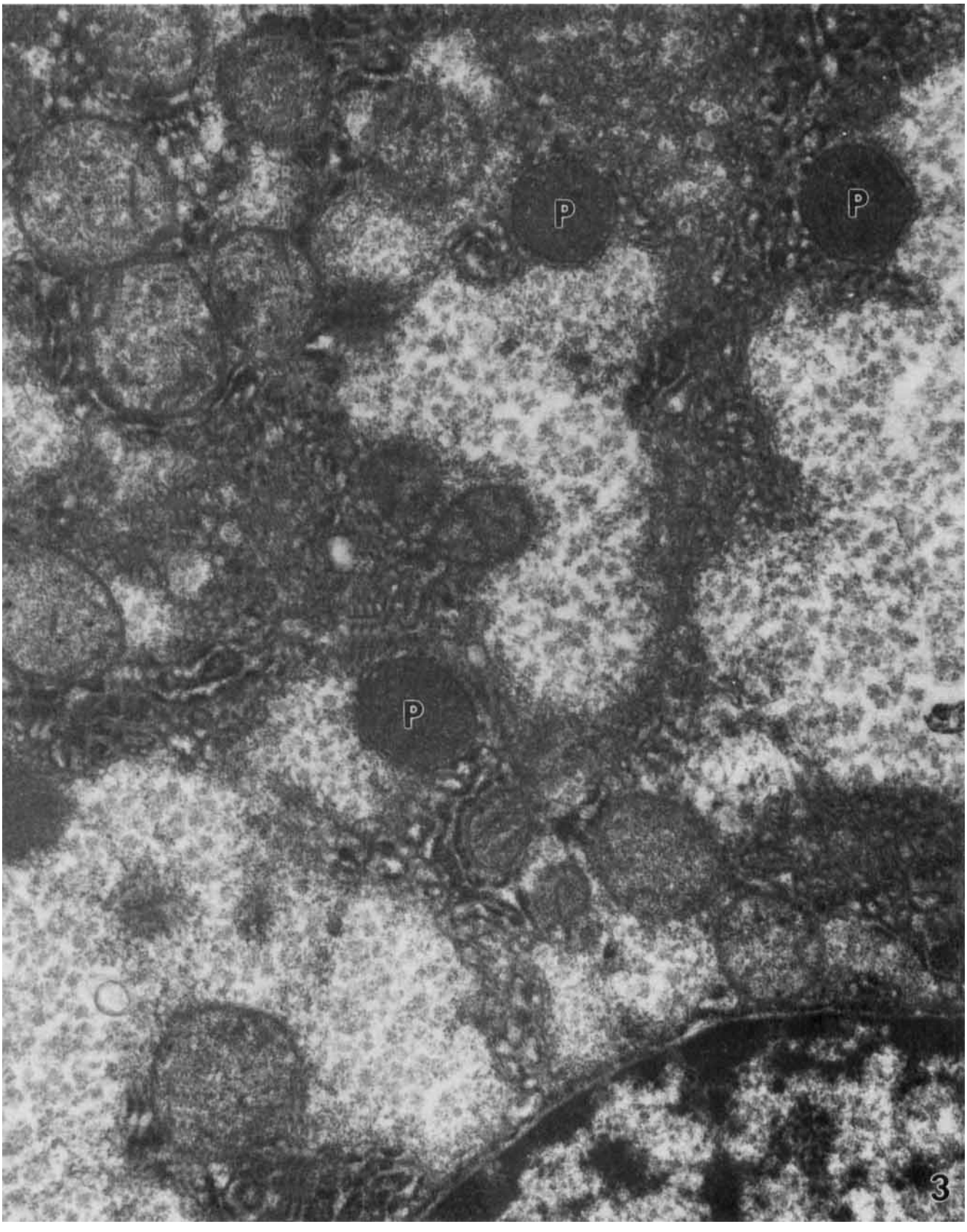

Figure 3. Hepatocyte from control primate liver cell, showing normal number of peroxisomes (P). 30 000×.

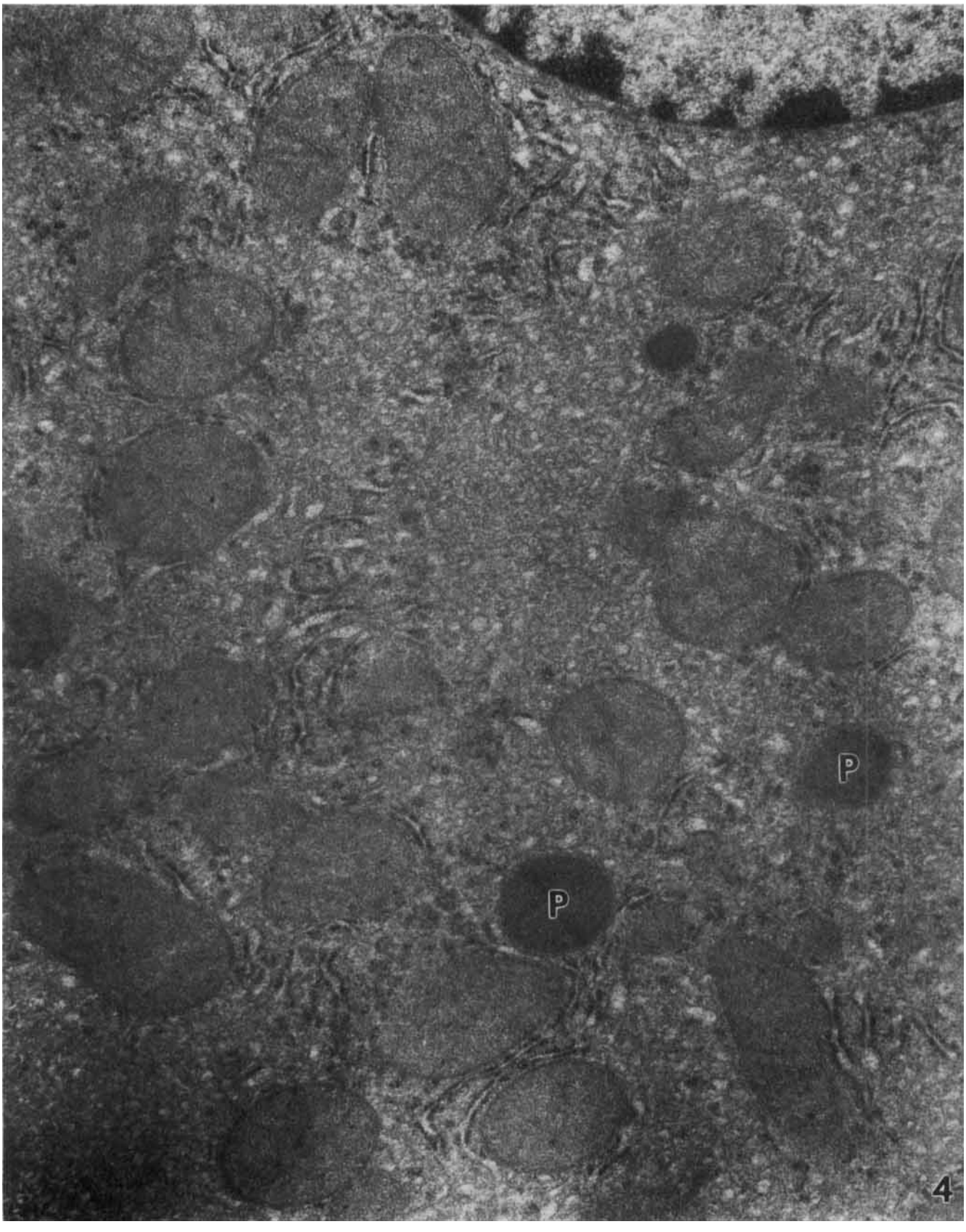

Figure 4. Hepatocyte from primate liver exposed to polyCTFE for 15 days, showing normal number of peroxisomes (P). 30 000×.

kg^{-1} polyCTFE had concentrations of trimer and tetramer, respectively, of 1.8 and 2.0 mg l^{-1} in blood and 70 and 100 mg l^{-1} in the liver. In rats exposed by inhalation to polyCTFE (0.250 mg l^{-1}, 6 h per day, 5 days per week), blood concentrations were 0.9 mg l^{-1} for trimer and 2.0 mg l^{-1} for tetramer. Liver concentrations were 20 and 54 mg l^{-1}.[12] Although liver concentrations were of the same order of magnitude in both species, there were significant differences in the effects on the liver. Rat liver showed a variety of cellular structural changes, which included an increase in smooth endoplasmic reticulum and mitochondrial swelling. Monkeys had only minor changes in the hepatocytes and only slight mitochondrial swelling, which was also seen in controls. The major difference in the two species was a five-fold increase in the number of peroxisomes and an increase in fatty acid beta-oxidation activity in rats.[12] The monkeys in this study had no such increase. The issue of peroxisomal proliferation warrants specific comment. The findings in this study are in contrast to the findings of Reddy *et al.*,[18] who induced peroxisomal proliferation in primates by increasing the dose of a hypolipidemic drug. Additionally, treatment with peroxisomal proliferators in rats has demonstrated an association with carcinogenesis, although the correlation in other species is equivocal.[15] The present study indicates that the extent of peroxisomal proliferation in response to proliferators may be species-specific. The increase in serum triglycerides (five-fold at the end of the study) was not expected based on rat data. Repeated oral dosing of rats with polyCTFE for 4 weeks resulted in no change in serum triglycerides.[24] The increase in serum triglycerides in monkeys suggests a distinct response to dosing with polyCTFE. Current knowledge of lipid metabolism in the Rhesus monkey and rats[25] does not support more than a quantitative difference. Since an increase in triglycerides reflects fat mobilization, this increase and the increase in BUN (11% over the last 8 days of the study) probably reflect mobilization of alternative energy sources as a result of the observed appetite suppression and liver glycogen depletion.

Based on this study, toxicity of polyCTFE in rats may not be an appropriate indicator of a potential human hazard. Additional studies on the mechanism of peroxisome induction and on the events leading to carcinogenesis are required to assess better the role of fatty acid carcinogenesis. At present, it is not clear whether the relative non-toxicity of short-term administration of polyCTFE in primates is due to reduced metabolism to a peroxisome proliferator or to a reduced sensitivity in the induction process.

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