PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 430–439 (1983)

Induction of Rabbit Renal Mixed-Function Oxidases by Phenobarbital: Cell Specific Ultrastructural Changes in the Proximal Tubule (41583)

GLENN F. RUSH, KEIZO MAITA, STUART D. SLEIGHT, AND JERRY B. HOOK

Department of Pharmacology and Toxicology and Department of Pathology, Center for Environmental Toxicology, Michigan State University, East Lansing, Michigan 48824

Abstract. Administration of phenobarbital (60 mg/kg) daily for 4 days to male rabbits resulted in induction of renal cytochrome P-450 (3.5-fold) and a corresponding increase in ethoxycoumarin-*O*-deethylase and benzphetamine-*N*-demethylase activity (17- and 4-fold, respectively). Kidney weight to body weight ratio and renal ethoxyresorufin-*O*-deethylase were not affected by phenobarbital pretreatment. Numerous focal areas of proliferation of smooth endoplasmic reticulum (SER) were evident in proximal tubule cells from phenobarbital treated rabbits while proximal tubular cells from control rabbits had only small and sparcely located aggregates of SER. Phenobarbital-induced SER proliferation was specifically localized to the S_3 segment of the proximal tubule. Proliferation was not observed in S_2 cells of the proximal tubule, cells of Henle's loop, distal tubules, or collecting tubules in rabbits pretreated with phenobarbital. These data demonstrate the biochemical heterogeneity of cell types within the proximal tubules of rabbits. Furthermore, induction of mixed-function oxidases specifically in S_3 cells of the proximal tubule may be of toxicological significance in the metabolic activation of certain nephrotoxicants.

A microsomal cytochrome P-450 mixed function oxidase (MFO) system is present in the kidney of a variety of species (1–8). This system appears to be similar to that described in the liver; purified renal cytochrome P-450 (P-450) from pig kidney requires both NADPH cytochrome P-450 reductase and a phospholipid component for catalytic activity in a reconstituted system (5). The renal P-450 system is dissimilar to the liver system in regard to substrate specificity and response to enzyme inducers (6, 7). In general, hepatic MFOs are inducible by both phenobarbital (PB) and 3-methylcholanthrene (3MC) whereas in the kidney, induction of MFOs varies with the species. Rat and guinea pig renal MFOs respond to 3MC but not to PB whereas rabbit and hamster renal MFOs are induced by both PB and 3MC pretreatment (8–10). In liver, induction of microsomal enzymes by PB or tetrachlorodibenzo-*p*-dioxin (TCDD) results in a massive proliferation of smooth endoplasmic reticulum (SER) whereas induction by 3MC results in only a moderate proliferation of SER (11, 12). Similarly, a close association exists between proliferation of SER and induction of microsomal enzyme activities in the rat kidney (13–15).

Renal MFOs are not uniformly distributed within the kidney but exhibit a cortico-papillary gradient; i.e., enzyme activity is greatest in the cortex (6, 13). Furthermore, in the kidney, chemically-induced proliferation of SER is remarkably cell-type specific. Administration of TCDD to rats results in SER proliferation that is localized primarily to the S_3 segment of the proximal tubule but not in S_1 cells or directly adjacent S_2 cells or any other cell type within the kidney (13). Similar findings have also been observed with dieldrin, CCl_4, and possibly 3MC (14–16).

Cell specific induction of renal MFOs has only been reported in the rat with chemical inducers of the polycyclic halogenated hydrocarbon-class. Since PB causes massive SER proliferation in the liver and markedly induces rabbit renal MFOs, it was of interest to determine the effect of PB on microsomal enzyme activity and tubular distribution of SER in the rabbit kidney. These results demonstrate differential tubular segmental proliferation of SER in the rabbit kidney from animals pretreated with PB similar to that observed in rats treated with TCDD (13).

Materials and Methods. *Animals and treatments.* Male New Zealand white rabbits (1.5–2.0 kg) were purchased from local suppliers, housed in individual cages in a temperature-, humidity-, and light- (12 hr light:12 hr dark) controlled room and provided food and water

0037-9727/83 $1.50

ad libitum. Single injections of PB (60 mg/kg) were administered in saline, ip, daily for 4 days. Control animals received saline alone.

Electron microscopy. Animals were stunned by a blow to the head and the right kidney immediately ligated and removed. A cannula was quickly placed in the abdominal aorta posterior to the left renal artery, the aorta anterior to the left renal artery was ligated, and a small incision was made in the renal vein. The left kidney was then fixed *in situ* by perfusion with 1.25% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) for 5 min. Afterward the kidney was removed and thin midsagittal strips prepared and stored in the same buffer. Kidney strips were diced in 1-mm^3 blocks, washed twice in Zetterqvist's buffer (pH 7.5), placed in 1% OsO_4 solution for 1.5 hr, and embedded into Epon–Araldite plastic after dehydration through graded-alcohol and propylene oxide washes. Thin sections were then prepared on a LKB-III ultratome and stained with uranyl acetate and lead citrate prior to examination on a Zeiss EM952 electron microscope operated at 60 kV. Identification of ultrastructural segments of proximal tubules was by the criteria established by Maunsbach (17).

Biochemical analysis. After removal, the right kidney was immediately weighed and placed in ice-cold 1.15% KCl. Renal inner medulla and papilla were carefully dissected away and the cortex minced in 100 m*M* phosphate buffer (pH 7.4) containing 0.25 *M* sucrose and 5.4 m*M* EDTA. Minced tissues were rinsed three times and homogenized in three volumes of the same solution using a Potter–Elvehjem homogenizer with a Teflon pestle followed by centrifugation at 10,000*g* for 30 min. The resulting supernatant was then centrifuged at 105,000*g* for 60 min. The pellet was resuspended in buffer, pH 7.4, and recentrifuged at 105,000*g* for 60 min. The microsomal fraction (the pellet) was resuspended in 66 m*M* phosphate buffer, pH 7.4, to a final concentration of 10 to 20 mg of protein/ml.

The final microsomal suspension was used for the determination of benzphetamine-*N*-demethylase (18), ethoxycoumarin-*O*-deethylase (19), and ethoxyresorufin-*O*-deethylase (20). Cytochrome P-450 concentrations were determined according to the method of Omura and Sato (21). Protein was quantified by the method of Lowry *et al.* (22).

Statistics. Data were analyzed with Student's *t* test (23). The 0.05 level of probability was used as the criterion of significance.

Results. Administration of 60 mg/kg PB for 4 days resulted in increased liver weight/body weight but no change in kidney weight/body weight (Table I). Animal weights were not altered by this dose of PB (data not shown). Rabbit renal cytochrome P-450 content was increased 3.5-fold and ethoxycoumarin-*O*-deethylase and benzphetamine-*N*-demethylase were increased 17- and 4.8-fold, respectively, by PB (Table I). Ethoxyresorufin-*O*-deethylase was not affected (Table I). Recovery of microsomal protein from kidney was not increased by phenobarbital (Table I).

Ultrastructural segmentation of rabbit kidney proximal tubules was clearly identified and tubular cell components and structures of each segment were similar to those previously described (17, 24–26). Kidneys from saline-pretreated rabbits had occasional small aggregates of SER only at the apical border

TABLE I. THE EFFECT OF PHENOBARBITAL ON ORGAN WEIGHTS AND RENAL MIXED-FUNCTION OXIDASES IN THE RABBIT[a]

	Control	Phenobarbital
Liver wt/body wt	2.54 ± 0.18	3.87 ± 0.17[b]
Kidney wt/body wt	0.33 ± 0.02	0.37 ± 0.05
Microsomal recovery (mg/gm kidney)	5.14 ± 0.50	4.69 ± 0.43
Ethoxyresorufin-*O*-deethylase (nmole/min/mg protein)	0.013 ± 0.002	0.016 ± 0.003
Ethoxycoumarin-*O*-deethylase (nmole/min/mg protein)	0.101 ± 0.030	1.701 ± 0.175[b]
Benzphetamine-*N*-demethylase (nmole/min/mg protein)	0.211 ± 0.013	1.017 ± 0.061[b]
Cytochrome P-450 (nmole/mg protein)	0.14 ± 0.02	0.49 ± 0.03[b]

[a] Rabbits were administered phenobarbital ip 60 mg/kg once daily for 4 days. Control animals received saline.
[b] Significantly different from controls ($P > 0.05$); $n = 4$.

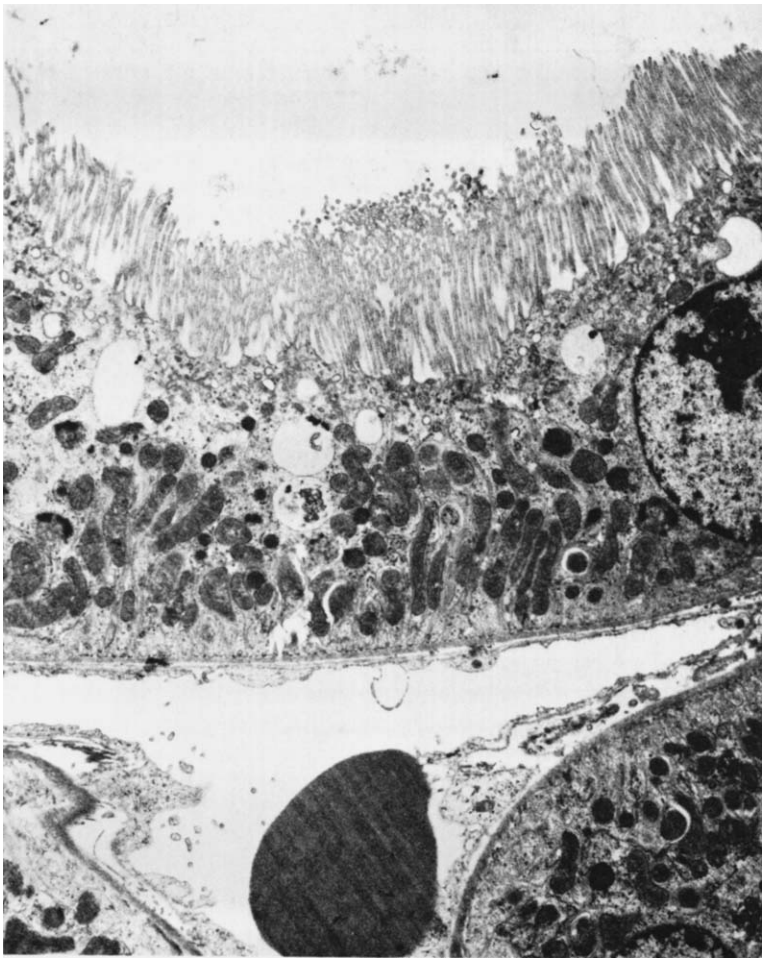

FIG. 1. Electronmicrograph of S_2 proximal tubular cells from a control rabbit. Cells are characterized as described in the text. ×5170.

or near the lateral boundaries of S_3 cells (Figs. 2, 5), but not in S_2 cells (Fig. 1). Kidneys from phenobarbital-treated rabbits had numerous focal areas of proliferation of SER in S_3 tubular cells (Figs. 3, 4). Renal SER proliferation in PB-pretreated rabbits appeared as aggregates in S_3 cells and appeared to have no apparent relationship to other subcellular organelles which were unchanged in their distribution and structure by phenobarbital (Figs. 3, 6). SER proliferation was not observed in S_2 segments of proximal tubules, distal tubules (Figs. 3, 4), Henle's loop, or collecting tubules (data not shown).

Discussion. Proliferation of SER after phenobarbital administration was restricted to the proximal tubule and, more specifically, the S_3 segment of the pars recta. Furthermore, induction of benzphetamine-*N*-demethylase and ethoxycoumarin-*O*-deethylase, which are typically increased by PB, was also evident (9). Since microsomal catalytic activity resides primarily with SER, a clear association was evident between the S_3 cells of the proximal tubule and renal drug metabolism. This close relationship of catalytic activity and SER proliferation has been demonstrated in liver from animals treated with PB (11) and in rat kidney from animals treated with TCDD (13). Ethoxyresorufin-*O*-deethylase is inducible by 3-methylcholanthrene, but not by PB, and likewise was not increased in rabbit kidney after

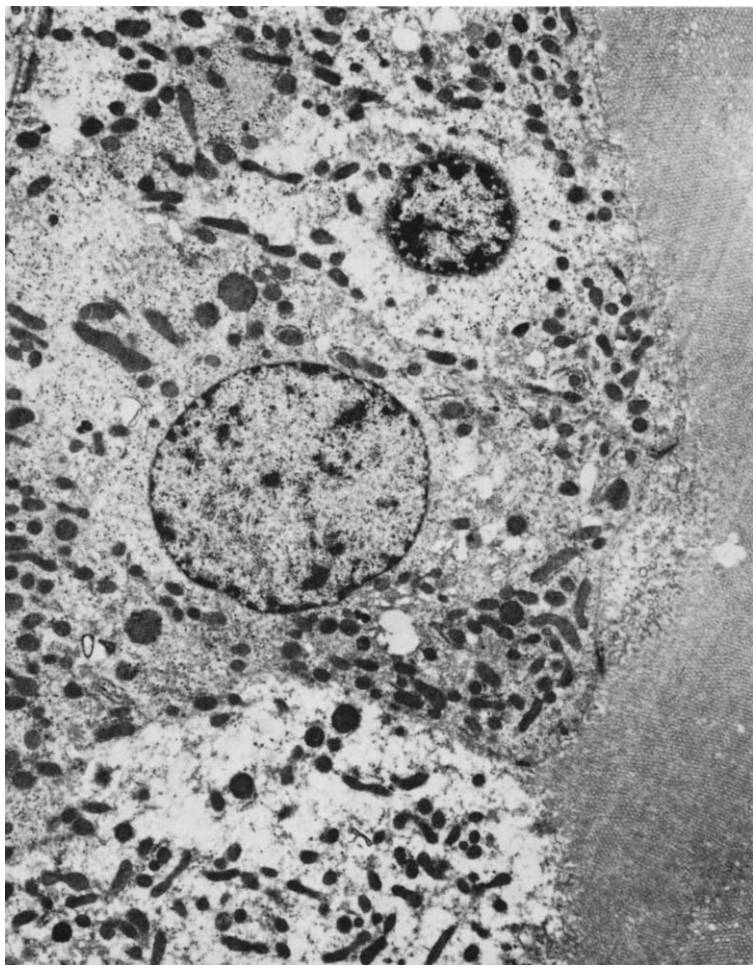

FIG. 2. Electronmicrograph of S_3 proximal tubular cell from a control rabbit. Cells are characterized as described in the text. ×4970.

PB (9). Data from the present experiments demonstrate that increased renal microsomal enzyme activity may be related to the cell-specific proliferation of SER after PB pretreatment in rabbits.

There is marked functional as well as morphological heterogeneity of rabbit proximal tubules. Para-aminohippurate is secreted at different rates along the proximal tubule ($S_2 < S_3 = S_1$) (27). Morphologically, brush border microvilli are longest in S_3 and shortest in S_2 and S_1; S_2 cells contain numerous apical vesicles and larger amounts of lysosomes in the cytoplasm (17). Mitochondria are long and perpendicular to the cellular axis in S_1 cells but become more randomly oriented in S_2 and S_3 cells. Basal invaginations of the plasma membrane are numerous and deep in S_1 cells and decrease in depth in S_2 and S_3 cells (28). Biochemical heterogeneity is also demonstrated by the apparent lack of PB-inducible SER in S_1 or S_2 cells whereas proliferation occurs in S_3 cells (Figs. 3, 4).

Recently, Dees *et al.* have described the occurrence and intercellular distribution of four hepatic cytochrome P-450 isozymes in rabbit kidney by using immunofluorescence (29). The major constitutive forms of P-450 in rabbit kidney were immunologically identical to hepatic form 2 (PB inducible) and form 3 (hepatic constitutive) and were localized primarily to the latter portion of the pars con-

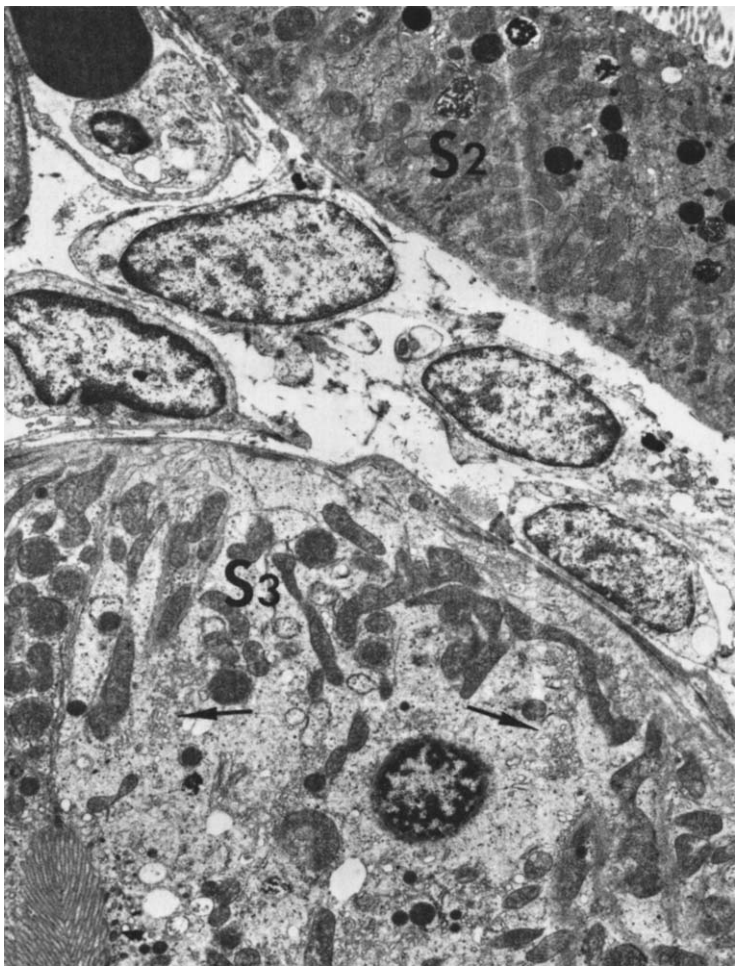

FIG. 3. Electronmicrograph of S_2 and S_3 proximal tubular cells from a rabbit pretreated with phenobarbital (60 mg/kg × 4 days). Cells are characterized as described in the text. Note prominent SER proliferation (arrows) in S_3 cells but not in S_2 cells. ×4970.

voluta (S_2) and the pars recta (S_3). Administration of PB did not alter the intercellular distribution of cytochrome P-450 but did increase the amount in the pars recta (29). The present observations on differential intercellular SER distribution in kidneys from rabbits pretreated with PB is in agreement with the observations of Dees *et al.* (29) and is similar to that observed in the rat administered TCDD (13). Although administration of PB increases microsomal yield and organ weight in the liver (data not shown), no such correlate was observed in the rabbit kidney (Table I). However, the proliferation of SER in rabbit kidney after PB administration was relatively small, localized in small aggregates and confined to a relatively small population of renal tubular cells and may therefore have gone undetected as an increase in microsomal recovery.

Microsomal preparations from kidney generally contain approximately one-tenth the P-450 concentration of the liver (7). For this reason, the significance of these enzymes in the kidney has been questioned. Although it is true that renal MFOs probably play a very minor role in the metabolism and elimination of the total body burden of xenobiotics, they may be important in the metabolic activation of a variety of nephrotoxicants. Necrosis of proximal tubules is a common toxic mani-

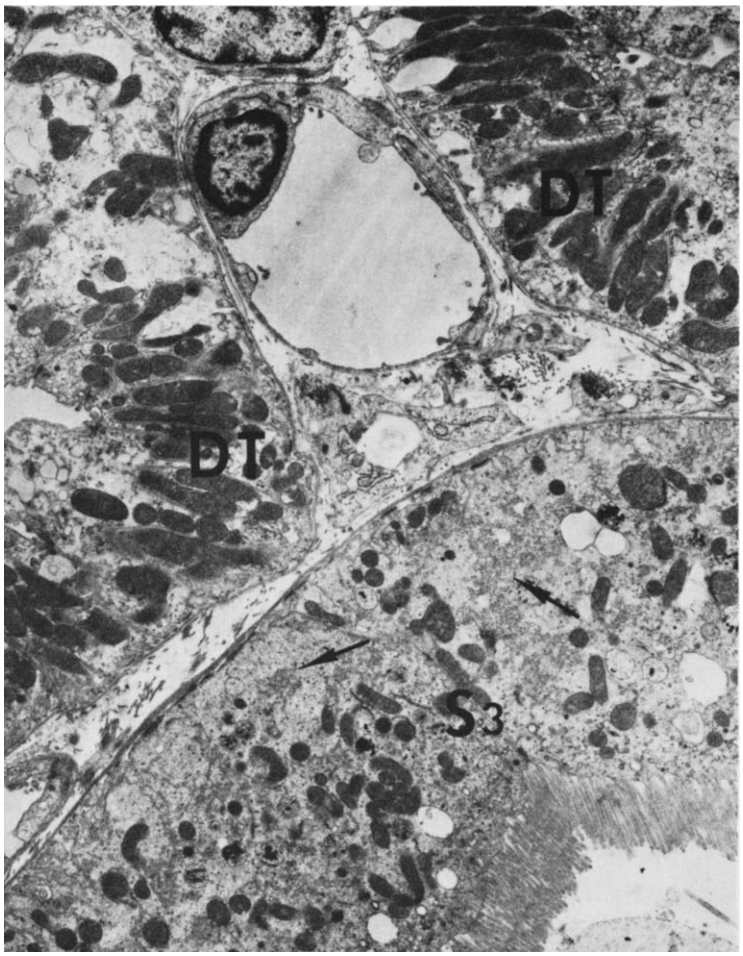

FIG. 4. Electronmicrograph of S_3 proximal tubular cells and distal tubular cell (DT) from phenobarbital (60 mg/kg × 4 days) pretreated rabbit. Note numerous SER aggregates (arrows) in S_3 cells but not in DT cell. Cells are characterized as described in text. ×4970.

festation of many chemicals that require metabolic activation for expression of toxicity (30). Furthermore, the observation that rabbit and rat renal MFOs are restricted to a specific cell type is important to renal toxicology since within the proximal tubule, there appears to be differential susceptibility of S_1, S_2, and S_3 cells to chemicals. For example, administration of large doses of acetaminophen to rats results in extensive necrosis confined primarily to the distal portions of the proximal tubule whereas glomeruli, early proximal convoluted tubules, and distal portions of the nephron are unaffected (31). Similar findings have also been reported for hexachlorobutadiene (32).

In conclusion, these data indicate an association between proliferation of SER and microsomal enzyme activity in the rabbit kidney after PB administration. SER proliferation was primarily localized to the S_3 segment of the proximal tubules as has been demonstrated in the rat treated with TCDD (14). This information has significance in the basic understanding of detoxifying and activating mechanism in the kidney by directly correlating biochemical changes with morphological alterations.

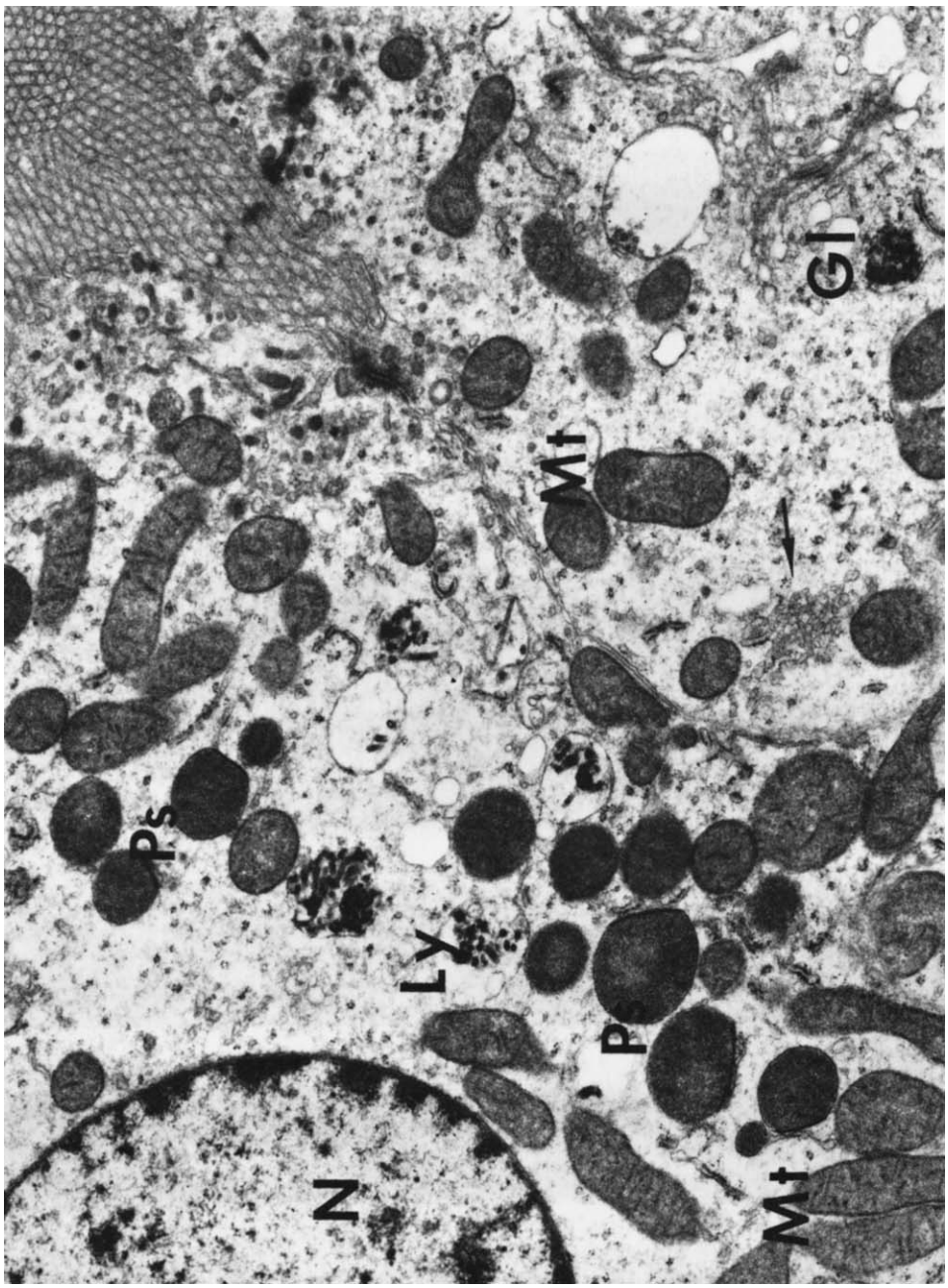

FIG. 5. Electronmicrograph of apical part of S_3 proximal tubular cell from a control rabbit. Numerous peroxisomes (Ps) are present together with lysosomes (Ly) and mitochondria (Mt). A small SER aggregate is seen at the lateral boundary (arrow). Nucleus (N), Golgi apparatus (Gl). ×16,300.

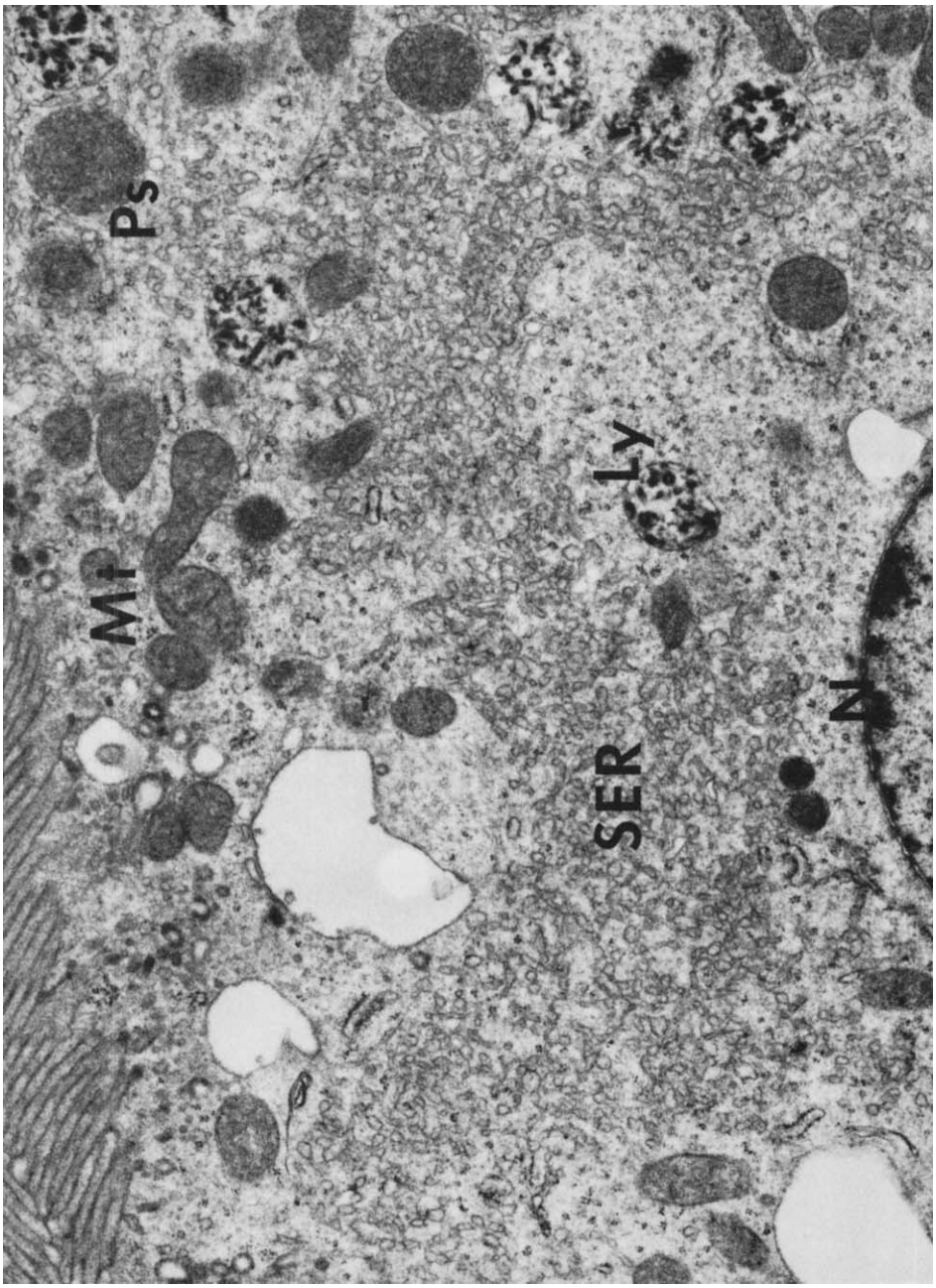

FIG. 6. Electronmicrograph of apical part of S_3 proximal tubular cell from a rabbit pretreated with phenobarbital (60 mg/kg × 4 days). Note massive SER proliferation. Nucleus (N), Mitochondria (Mt), Lysosome (Ly), peroxisome (Ps). ×16,300.

This investigation was supported in part by the National Institute of Environmental Health Sciences Grant ES00560. Dr. Rush is the recipient of a NIEHS Postdoctoral Fellowship No. ES05243. We wish to thank V. Adler for her expert technical assistance and D. Hummel for her help in manuscript preparation.

1. Orrenius S, Ellin A, Jakobsson SV, Thor H, Cinti DL, Schenkman JB, Estabrook RW. The cytochrome P-450-containing mono-oxygenase systems of rat kidney cortex microsomes. Drug Metab Disp **1**:350–356, 1973.
2. Ellin A, Jakobsson SV, Schenkman JB, Orrenius S. Cytochrome P-450$_k$ of rat kidney cortex microsomes: its involvement in fatty acid ω- and (ω-1)-hydroxylation. Arch Biochem Biophys **150**:64–71, 1972.
3. Jakobsson SV, Cinti DL. Studies on the cytochrome P-450-containing mono-oxygenase system in human kidney cortex microsomes. J Pharmacol Exp Ther **185**:226–234, 1973.
4. Litterst CL, Mimnaugh EG, Reagan RL, Gram TE. Comparison of in vitro drug metabolism by lung, liver and kidney of several common laboratory species. Drug Metab Disp **3**:259–165, 1975.
5. Okita RT, Parkhill LK, Yasukochi Y, Masters BSS. The ω and (ω-1)-Hydroxylase activities of prostaglandins A_1 and E_1 and lauric acid by pig kidney microsomes and a purified kidney cytochrome P-450. J Biol Chem **256**:5961–5964, 1981.
6. Zenser TV, Mattammal MB, Davis BB. Differential distribution of the mixed function oxidase activities in rabbit kidney. J Pharmacol Exp Ther **207**:719–725, 1978.
7. Jones DP, Orrenius S, Jakobson SW. Cytochrome P-450-linked monooxygenase systems in the kidney. In: Gram TE, ed. Extrahepatic Metabolism of Drugs and Other Foreign Compounds. New York/London, Spectrum, pp123–158, 1980.
8. Uehleke H, Greim H. Stimulierung der oxidation von fremdstoffen in nierenmikrasomen durch phenobarbital. Naunyn-Schmiedebergs Arch Pharmakol Exp Pathol **261**:151–161, 1968.
9. Rush GF, Hook JB. Selective inhibition of rabbit renal mixed function oxidases by metyrapone and α-naphthoflavone. Fed Proc **40**:637, 1981.
10. Rush GF, Smith JH, Hook JB. Induction of hepatic and renal mixed function oxidases (MFO) in the hamster and guinea pig. Fed Proc **41**:1638, 1982.
11. Orrenius S, Ericsson JLE, Ernster L. Phenobarbital-induced synthesis of the microsomal drug metabolizing enzyme system and its relationship to the proliferation of endoplasmic membranes. A morphological and biochemical study. J Cell Biol **25**:627–639, 1965.
12. Fowler BA, Lucier GW, Brown HW, McDaniel OS. Ultrastructural changes in rat liver cells following a single oral dose of TCDD. Environ Health Perspect **5**:141–148, 1973.
13. Fowler BA, Hook GER, Lucier GW. Tetrachlorodibenzo-*p*-dioxin induction of renal microsomal enzyme systems: Ultrastructural effects on pars recta (S_3) proximal tubule cells of rat kidney. J Pharmacol Exp Ther **203**:712–721, 1977.
14. Strika GE, Smuckler EA, Kohnan PW, Wagle RB. Structural and functional changes in rat kidney during CCl_4 intoxication. Amer J Pathol **53**:769–789, 1968.
15. Fowler BA. The morphologic effects of dieldrin and methyl mercuric chloride on pars recta segments of rat kidney proximal tubules. Amer J Pathol **69**:163–174, 1972.
16. Wattenberg LW, Leong JL. Histochemical demonstration of reduced pyridine nucleotide dependent polycyclic hydrocarbon metabolizing systems. J Histochem Cytochem **10**:412–420, 1962.
17. Maunsbach AB. Observation on the segmentation of the proximal tubule in the rat kidney. Comparison of results from phase contrast, fluorescence and electron microscopy. J Ultrastruct Res **16**:239–258, 1966.
18. Prough RA, Ziegler DM. The relative participation of liver microsomal amine oxidase and cytochrome P-450 in demethylation reactions. Arch Biochem Biophys **180**:363–373, 1977.
19. Ullrich V, Weber P. The *O*-dealkylation of 7-ethoxycoumarin by liver microsomes. A direct fluorometric test. Hoppe-Seylers Z Physiol Chem **353**:1171–1177, 1972.
20. Johnson EF, Schwab GE, Muller-Eberhard V. Multiple forms of cytochrome P-450: Catalytic differences by two homogeneous forms of rabbit cytochrome P-450. Mol Pharmacol **15**:708–718, 1979.
21. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. The evidence for its hemoprotein nature. J Biol Chem **239**:2370–2378, 1974.
22. Lowry OH, Rosebrough NJ, Farr AJ, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193**:265–275, 1951.
23. Steel RGD, Torrie JH. Principles and Procedures of Statistics. New York, McGraw-Hill, pp 194–205, 1960.
24. Beard MG, Novicoff AB. Distribution of peroxisomes (microbodies) in the nephron of the rat. A cytochemical study. J Cell Biol **82**:501–518, 1968.
25. Griffith LD, Bulgen RE, Trump BF. The ultrastructure of the functional kidney. Lab Invest **16**(2):220–246, 1967.
26. Jacobson ND, Jorgensen A. Ultrastructural observation on the pars descendens of the proximal tubule in the kidney of the male rat. Z Zellforsh **136**:479–499, 1973.
27. Grantham JJ, Irish J. Organic acid transport and fluid secretion in the pars recta (PST) of the proximal tubule. In: Vogel HG, Ullrich KJ, ed. New Aspects of

Renal Function. Amsterdam, Excerpta Medica, pp83–87, 1978.

28. Venkatachalam MA, Bernard DB, Donahoe JF, Levinsky NG. Ischemic damage and repair in the proximal tubule: Differences among S_1, S_2 and S_3 segments. Kidney Int **14**:31–49, 1978.
29. Dees JH, Masters BSS, Muller-Eberhard U, Johnson EF. Effect of 2,3,7,8-tetrachlorodipenzo-*p*-dioxin and phenobarbital on the occurrence and distribution of four cytochrome P-450 isozymes in rabbit kidney, lung and liver. Cancer Res **42**:1423, 1982.
30. Hook JB, McCormack KM, Klowe KM. Biochemical mechanisms of nephrotoxicity. In: Hodgson E, Bend JR, Philpot RM, ed. Reviews in Biochemical Toxicology. New York, North Holland, Vol 1pp53–79, 1979.
31. McMurtry RJ, Snodgrass WR, Mitchell JR. Renal necrosis, glutathione depletion and covalent binding after acetaminophen. Toxicol Appl Pharmacol **46**:87–100, 1978.
32. Lock EA, Ishmael J. The acute toxic effects of hexachloro-1:3-butadiene on the rat kidney. Arch Toxicol **43**:47–57, 1979.

Received September 2, 1982. P.S.E.B.M. 1983, Vol. 172.