Structural and Functional Changes in Rat Kidney During CCl_4 Intoxication

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ALTERATIONS in kidney structure and function are frequently encountered in severe liver disease,[1] but the relationship between the hepatic and renal disorders is not clear.[2,3] The possibilities are that the agent(s) precipitating the liver disease may affect the kidney directly [1, 3-5] or that the renal lesions develop as a secondary phenomenon to the altered liver function.[3] Administration of carbon tetrachloride (CCl_4) to rodents results in a reproducible liver necrosis and provides a model system in which to study these interactions. The following series of experiments were undertaken to define the early renal lesion encountered in CCl_4 intoxication.

Materials and Methods

Male Sprague-Dawley rats (200–260 gm.) were maintained in metabolic cages with free access to Purina Lab Chow and water. Prior to the experiments the animals were fasted for 16–18 hr., but were provided water ad libitum. CCl_4 (at a dosage of 0.25 ml. CCl_4 per 100 gm. body weight) in an equal volume of mineral oil was given by gastric tube without anesthesia. Control animals received 0.5 ml. mineral oil per 100 gm. body weight. All experiments were started in the morning to reduce diurnal variation. Access to food was provided 2 hr. after intubation. Urinary output and water intake were recorded every 12 hr. Two experimental and two control animals were sacrificed by exsanguination after ether narcosis at 2, 6, 12, 24, and 120 hr. following intubation. A second group of animals was treated identically except for a 12-hr. period of dehydration before intoxication, and with sacrifice at 12, 24, and 36 hr.

Morphologic Studies

Coronal sections of the kidneys were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Additional fragments were quickly frozen in liquid nitrogen and sectioned in a cryostat. Sections from the paraffin-embedded tissue were stained with hematoxylin and eosin, Masson's trichrome, and the periodic acid-Schiff technique. The frozen sections were stained with oil red O, with hexazotized pararosanilin for acid phosphatase, and with naphthol AS-MX for alkaline phosphatase.

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Small fragments of renal cortex and medulla were placed in 6.25% glutaraldehyde (4° C.), buffered with 0.01 M cacodylate (pH 7.4), and fixed for 6 hr. The fragments were washed with the same buffer made isotonic with sucrose, postfixed in 2% osmium tetroxide buffered with *s*-collidine (pH 7.4) for 2 hr., dehydrated in a graded alcohol series, and embedded in Epon.[6] Sections (0.5–1.0 μ thick) were obtained with a Porter-Blum microtome to determine the portion of the proximal tubule [7] contained in the section and the level of the cortex. Thin sections were stained with uranyl acetate [8] and/or lead tartrate [9] and examined in an RCA EMU 3G electron microscope.

Chemical and Enzyme Studies

Urine collected from metabolic cages, and bladder urine and serum obtained at sacrifice were analyzed for urea,[10] protein,[11] creatinine,[10] $Na+$,[10] and $K+$.[10] The osmolality of serum and urine were measured by the method of Bowman *et al.*[12] with an Advanced Instrument osmometer.

A series of animals, consisting of groups of 3 experimental and 1 control animal, were sacrificed at various time intervals by a sharp blow to the head. One kidney was removed, stripped of its capsule, weighed, and dried to a constant weight in a 60° C. oven. The ratio of wet weight to dry weight was so obtained. The second kidney was removed, weighed, and homogenized in four volumes of cold 0.25 M sucrose. All subsequent operations were performed at 4° C. unless otherwise specified. The homogenate was centrifuged at 800 *g* for 10 min. to sediment unruptured cells, nuclei, and debris. To prepare mitochondria and lysosomes, aliquots of the postnuclear fraction were centrifuged at 12,500 *g* for 20 min. The sediment was washed twice with four volumes of 0.25 M sucrose and recentrifuged. The supernatant fluids from this step were combined and centrifuged at 105,000 *g* for 60 min. The 12,500 *g* and 105,000 *g* pellets were suspended in 0.25 M sucrose, and their protein concentration measured by the Lowry *et al.*[11] technique using bovine serum albumin as a standard. The protein concentration of the mitochondrial-lysosomal and microsomal suspensions, and the 105,000 *g* supernatant fluid, were adjusted to 4 mg./ml. with 0.25 M sucrose.

Enzyme Assays

Glucose-6-phosphatase activity was determined on 0.1-ml. aliquots of the various fractions by the method of Appelmans *et al.*[13] The incubation mixture contained, in a total volume of 0.5 ml.: 30 μmoles maleate buffer pH 6.5, 10 μmoles of glucose-6-phosphate, and 0.4 mg. of the cell fractions. The mixtures were incubated aerobically at 37° C. for 15 min., and the reaction was stopped by the addition of 2 ml. of 10% trichloracetic acid (TCA). Inorganic phosphorus was determined in the TCA-soluble fraction by the Fiske-SubbaRow method.[14] Controls included incubation mixtures to which TCA was added at zero time and mixtures incubated without the addition of glucose-6-phosphate.

Acid and alkaline phosphatase levels were determined by the methods of Berthet *et al.*[15] The incubation mixture contained, in a total volume of 1 ml.: 0.4 mg. of the cell fractions, 0.24 μmoles of paranitrophenylphosphate, and either 90 μmoles Tris buffer pH 9.0, or 90 μmoles of acetate buffer pH 5.2. The mixture was incubated aerobically at 37° C. for 15 min. and the reaction terminated by the addition of 300 μmoles K_2HPO_4. Controls included mixtures without the addition of substrate or those to which K_2HPO_4 was added at zero time.

Succinic dehydrogenase was determined using 2,6-dichlorophenol-indophenol (DCPIP) as the hydrogen acceptor. The reaction mixture contained, in a total volume of 1.6 ml.: 0.2 mg. of the various subcellular fractions, 20 μmoles potassium phosphate buffer pH 7.2, and 70 μmoles DCPIP. The mixtures were incubated

aerobically at 37° C. for 10 min., and the reaction was stopped by immersing the tubes in ice water. Cold distilled water, 2 ml., was added to each tube, and the absorbance of the resulting solution was measured at 500 mμ. Blanks included reaction mixtures maintained at 0° C. and those to which exogenous substrate was not added.

Preliminary experiments were performed to determine optimal protein concentration and reaction times for each of the assays.

Results

Gross Changes

A progressive increase in size and paleness of the kidney was noted during the first 2 days and was maximal at 48 hr. At this time the width of the cortex was approximately 1.0 mm. greater in kidneys from the treated group of animals. Changes in wet or dry weight were not significant until 36 hr. At this time, an increase in water content of the kidney (Table 1) and maximal diuresis was present.

Table 1. Comparison of Wet and Dry Weights of Kidneys

After CCl_4 (hr.)	Rats (No.)	Wet weight (gm./100 gm. BW)	Dry weight (gm./100 gm. BW)
Control	6	0.426 ± 0.01	0.091
1	3	0.419 ± 0.014	0.092
3	6	0.394 ± 0.014	0.099
6	6	0.413 ± 0.014	0.098
12	8	0.407 ± 0.01	0.098
24	8	0.439 ± 0.017	0.100
36	9	0.554 ± 0.01	—
48	5	0.520 ± 0.035	0.095
96	3	0.552 ± 0.047	—

Adult Sprague-Dawley rats were given 0.25 ml. CCl_4 per 100 gm. body weight (BW) and sacrificed at the intervals specified above. One kidney was rapidly removed, stripped of capsule and fat, and weighed. It was then diced and dried to a constant weight in a 60° C. oven.

Light Microscopy

Slight swelling of the epithelial cells of the proximal convoluted tubule and small vacuoles lying near the base of the cells became apparent by 24 hr. The cell swelling and vacuolation persisted at 48 hr. and had disappeared at 120 hr. At no time was there a detectable change in the amounts of lipid or acid and alkaline phosphatases present, as measured by histochemical means.

Electron Microscopy

The structure of the nephron in control animals was similar to that described by others (Fig. 1) except for differences which might be attributed to fixation.[7,16-19]

Alterations related to CCl_4 administration were limited to the proxi-

mal tubule and appeared to involve primarily the middle and lower segments. At 2 hr. after administration of CCl_4, the mitochondria appear swollen (Fig. 2), the cristae lacked the smooth contours of the normals, and the matrix was less dense. The mitochondria also appeared more rounded and irregular in configuration. Occasional small aggregates of smooth-surfaced membranes were noted: they were located mainly near the base of the cell and had no consistent organizational pattern. Rarely, there were ribosomes attached to membranes at the periphery of the aggregates. The profiles were clearly distinct from the paramembranous tubular system.[17] Microbodies were often seen near the profiles, but no connections were noted.

At 12 hr. after exposure, the mitochondrial changes were still present, and cell swelling was seen (Fig. 3). The microvilli were widened and club-shaped. Basilar interdigitations were absent in many of cells, but were present in the cells of adjacent, less affected tubules. Large numbers of the membranous profiles described above were seen scattered throughout the cell with some tendency to be more concentrated near the lateral cell margins (Fig. 3); they were occasionally seen adjacent to microbodies. Ribosomes could be seen attached to membranes at the periphery of many of the profiles. Some of the spaces defined by the membranes were irregular and dilated. The membranes had no discernible pattern of organization. A normal complement of other cell organelles was present.

At 24 hr. after intoxication, the mitochondrial changes were minimal, but cell swelling and the number of membranous profiles had increased (Fig. 4). The latter remained loosely packed, but their size had increased considerably. They frequently were seen in cells which had relatively normal basilar interdigitations (Fig. 5). The paramembranous tubular system was prominent at all time periods when large aggregates of membranous profiles were present. More frequently, ribosomes were seen attached to the membranous profiles at the periphery, especially of those which were more compact. Autophagic vacuoles containing cell debris were prominent in many cells containing the larger aggregates of membranes. Many cells contained large single-membrane-bound vacuoles lying near the base of the cell. Most of the vacuoles contained no electron-dense material, but an occasional irregular mass of material could be found contained therein (Fig. 5).

At 48 hr. after exposure the cell swelling was less apparent, and the membranous profiles were more compact and numerous (Fig. 6).

At 5 days no differences could be seen between experimental and control animals.

Urine and Serum Studies

The urine volume of control animals remained at approximately 6 ml. per rat per 24 hr. throughout the experimental period (Table 2). In the

Table 2. Serum and Urine Studies after CCl_4 Intoxication

After CCl_4 (hr.)	Mean urine vol. (ml./rat/12 hr.)		Urinary Na^+ (μEq./12 hr.)		Urinary K (μEq./12 hr.)		Serum creatinine (mg./100 ml.)	
	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.
2	—	—	250	170	620	350	—	1.15
12	2.75	4.0	200	240	300	360	0.95	1.7
24	3.0	12.25	260	460	430	490	0.90	1.3
48	4.5	15.75	390	1180	690	970	1.05	1.2
120	4.25	3.75	730	630	1690	700	1.05	1.1

Adult Sprague-Dawley rats were given 0.25 ml. CCl_4 per 100 gm. body weight and were placed in metabolic cages. Urine was collected at 12-hr. intervals and at sacrifice. Serum and bladder urine specimens were taken at sacrifice.
Cont., control animals; Exp., experimental animals.

experimental group urine output rose rapidly between 12 and 48 hr. and returned to control levels by 5 days (Table 2). The relation between water intake and urine output is shown in Table 3. The mean urine

Table 3. Effect of Water Deprivation

After CCl_4 (hr.)	Urine vol. (ml./rat)		Water intake (ml./rat)		Urinary osmolality	
	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.
−12	5	5	—	—	1750	1750
0	1.5	2.0	—	—	—	—
12	6	3.6	26	4.3	900	1150
24	5	10	20	1.5	1700	1300
36	5	13.2	32	29	2325	690

Adult Sprague-Dawley rats were housed in metabolic cages. After 12 hr. of water deprivation, they were given 0.25 ml. CCl_4 per 100 gm. body weight.
Cont., control animals; Exp., experimental animals.

volume of the treated group during the first 12 hr. was decreased. During the ensuing 24 hr. it rose rapidly, despite a relatively low water intake; the osmolality of the urine collected from the bladder at sacrifice decreased, and the total $Na+$ excreted was greater than the $K+$ and significantly exceeded control values (Table 2). The experimental group experienced a weight loss averaging 10% of the starting value, while the weight of the controls either remained stable or increased. Serum osmolality, sodium, potassium, and protein were unchanged. Serum creatinine peaked at 12 hr., while the blood urea nitrogen was maximal at 24 hr. Both values were normal at 5 days. Total serum bilirubin was elevated at 12–48 hr. and by 5 days had returned to control values.

Enzyme Studies

The succinic dehydrogenase activity (optical density, O.D. ÷ milligrams of protein) of the postnuclear supernatant was depressed to approximately 60% of the control values at 6 hr., returning to control levels or above by 36 hr. (Table 4). Similar results were found when the mitochondrial-lysosomal fraction [13] was used in place of the cruder fraction.

No change was seen in acid or alkaline phosphatase activity in either the postnuclear homogenate or the various subcellular fractions (Table 4). The relative amount of free vs. total activity was similar for treated and control groups.

Table 4. Enzyme Studies after CCl_4 Intoxication

After CCl_4 (hr.)	Glucose-6-phosphatase (γ P/mg. protein)	Succinic dehydrogenase (O.D./mg. protein)	Acid phosphatase (treated:control)
Control	35.7 ± 2.3	0.563 ± 0.041	—
1	40.9	0.659 ± 0.086	—
3	39.7 ± 0.6	0.628 ± 0.089	—
6	37.1 ± 2.65	0.342 ± 0.088	—
12	35.8 ± 5.8	0.430	1.07
24	37.1 ± 3.0	0.387 ± 0.047	0.82
36	60.6 ± 15.9	0.650	1.06
48	52.8	0.935 ± 0.365	0.84

Adult Sprague-Dawley rats were given 0.25 ml. CCl_4 per 100 mg. body weight and were sacrificed at the above specified intervals. Kidneys were rapidly removed, homogenized in 0.25 M sucrose, and the postnuclear supernate was analyzed.

Glucose-6-phosphatase levels were not significantly altered until 36 hr., after which a substantial increase was noted (Table 4).

Discussion

Reversible alterations occur in renal proximal tubular epithelium during the initial 48 hr. following CCl_4 administration. At the level of light microscopy the lesion is subtle, but distinct changes are recognizable in electron micrographs. The earliest morphologic change is seen in mitochondria, followed by cellular swelling, which is manifested by loss of basilar interdigitations and swollen microvilli. The appearance of large aggregates of smooth-surfaced membranous profiles occurs later.

One of the major functions of the proximal tubule is the active resorption of sodium ions from the tubular lumen.[20] Water follows passively reducing the glomerular filtrate to 20% of its volume in this tubular segment. The selective reabsorption of sodium ions is an energy-requiring process, and is believed to occur at the cell surface.[21] During the time at which there is impairment in sodium ion and water conservation, the

microvilli of the proximal tubular cells are clubbed, the basilar interdigitations are fewer in number, and the cells appear swollen. Prior to the onset of cell edema, mitochondrial alterations are seen. Since the prime source of cell energy is believed to be the mitochondria, these data suggest that the observed functional changes are related to decreased mitochondrial energy production. Consistent with this is the finding that one mitochondrial enzyme, succinic dehydrogenase, shows reduced activity parallelling altered mitochondrial morphology; in addition, it returned to and above normal with restoration of normal structure. The observations parallel the return of normal tubular function. The complexity of the relationship of mitochondrial structural and functional change and energy utilization is shown by the maintenance of renal ATP levels during this period.[22]

Aggregates of smooth-surfaced structures were noted early in the course of the intoxication and became more prominent with time. Concomitantly, there was an increase in one enzyme, glucose-6-phosphatase, believed to be linked to cell membranes. The roles that these structures might play in cellular and renal functions is not clear. The increased glucose-6-phosphatase activity parallels the appearance of these structures, and suggests that they possess at least this enzyme in common with other smooth-surfaced membranes. Their presence is not unique; they have been found in liver and kidney following intoxication with various organic compounds.[23-27]

The sequence of changes in the liver following CCl_4 intoxication is considerably different from that in the kidney.[28] The first change in the liver involves the rough-surfaced endoplasmic reticulum. Mitochondrial injury and cell swelling occur later, as does proliferation of the smooth-surfaced endoplasmic reticulum. The presence of an early functional mitochondrial change in the liver has been the subject of dispute. There is considerable evidence that CCl_4 per se is not responsible for the liver lesion, but that it must be metabolized to produce the observed cellular alterations.[28] The fact that morphologic and functional changes do not coincide with tissue CCl_4 levels supports this concept. A similar discordance exists in the kidney lesion—i.e., the lesion follows by hours the elimination of CCl_4 from the body.

The presence of an early lesion which has resolved considerably during a time when liver necrosis is becoming maximal suggests that the renal lesion is probably not a secondary phenomenon of the liver injury. The serum bilirubin levels were modest during the genesis of the renal lesion and remained elevated when the kidney lesion had resolved. This asynchrony suggests that the tubular lesion was related to CCl_4.

It does not exclude the possibility that the toxic substance is a metabolite of CCl_4 which could have been produced in the liver. The lesion has many of the morphologic and functional characteristics of chemicals causing more severe abnormalities, but necrosis was not apparent at the dose level employed.[2,4,17,24-26,29-33]

Summary

CCl_4 produces an early, reversible renal lesion limited to the proximal tubule. The first change occurs in the mitochondria, followed by cell swelling and proliferation of the smooth-surfaced endoplasmic reticulum. There is a parallel decrease in the ability to conserve sodium ions and water and in succinic dehydrogenase activity. The significance of these findings is discussed.

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Legends for Figures

All materials prepared for electron microscopy were fixed in 6.25% glutaraldehyde and postfixed in 2% OsO_4. The illustrations are of sections stained with uranyl acetate and lead tartrate.

Fig. 1. Proximal tubular cell near midportion of proximal tubule; from control animal. Cell interdigitations are numerous and extend to the level of the macula adherentes. Mitochondria are long, oval structures with parallel cristae. Lysosomes are numerous. × 12,470.

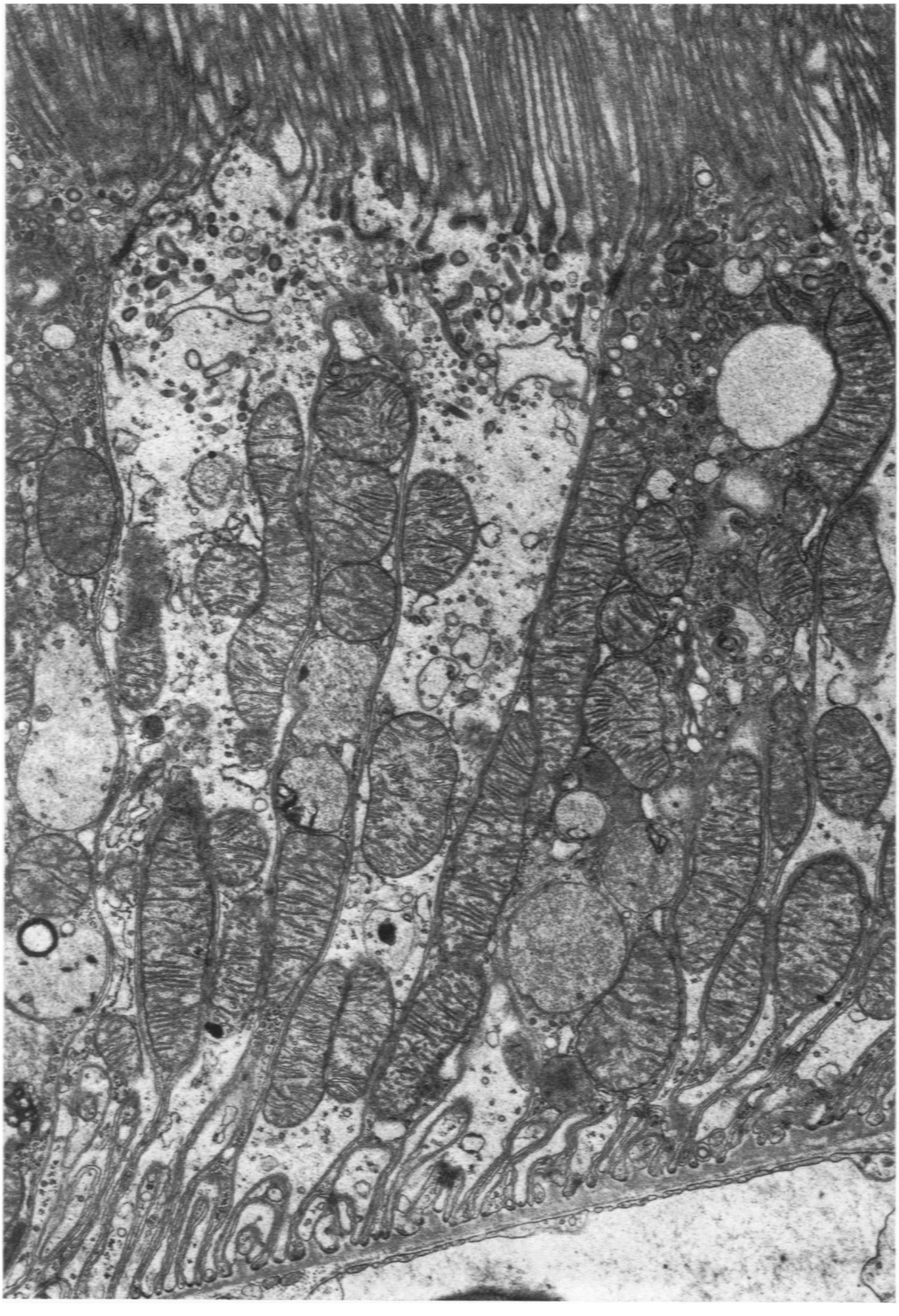

Fig. 2. Midproximal tubule region 2 hr. after single dose of CCl_4. Mitochondria are rounded, and parallel arrays of cristae are disrupted. Mitochondrial matrix is more dense, and there is dilation of the intercristal space. × 16,500.

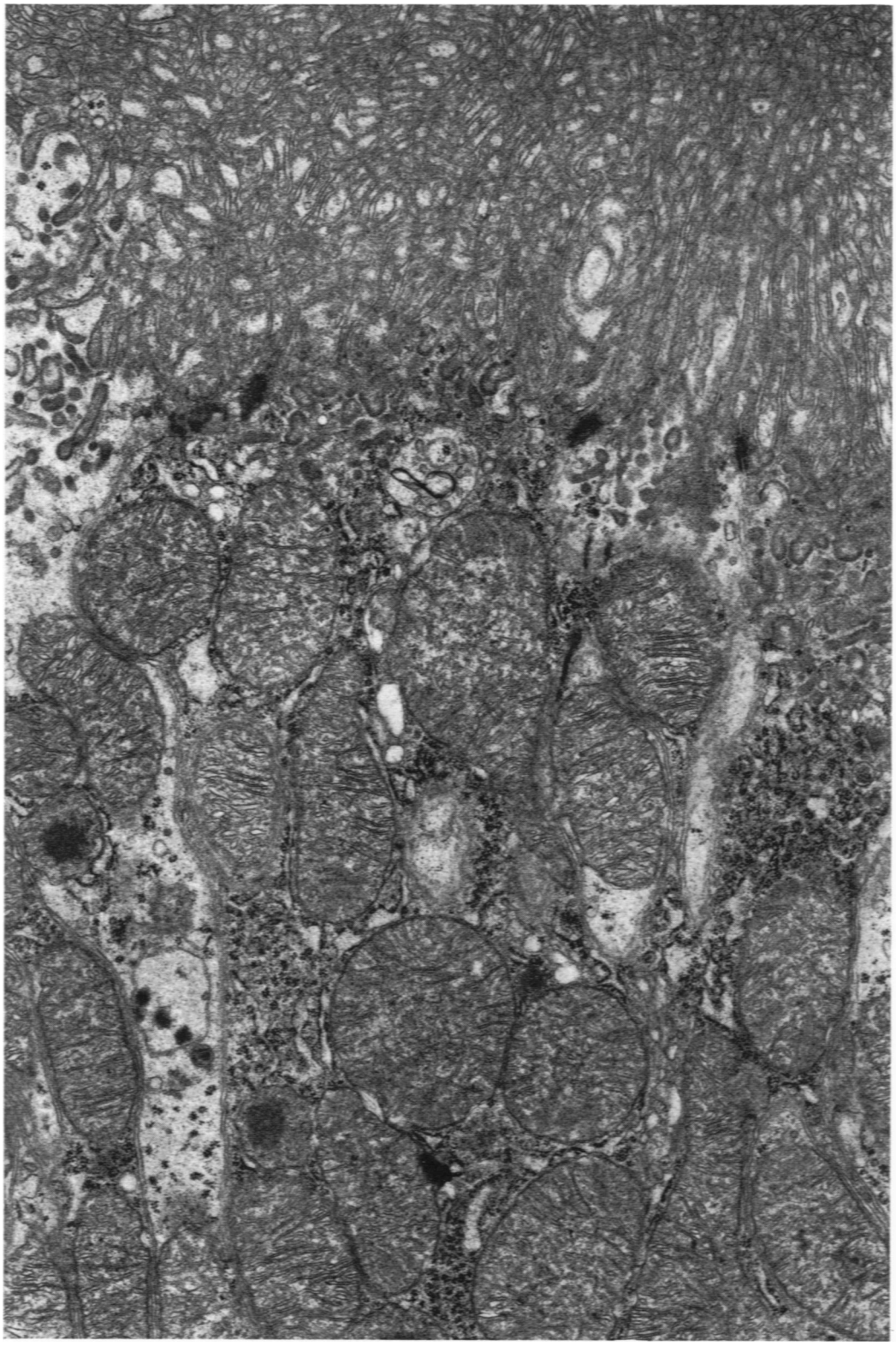

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Fig. 3. Lower midproximal tubule 12 hr. after 0.25 ml. CCl_4. Mitochondria are rounded; matrix shows irregular densities, and cristae are separated and shortened. Microvilli are widened. Basilar cellular interdigitations are relatively sparse. Loose aggregates of smooth-surfaced membranes lie in all three cells. In the cells in the center and to the right, microbodies are in close approximation to smooth-surfaced aggregates. Paramembranous tubular system appears at lateral margins of middle cell. × 12,470.

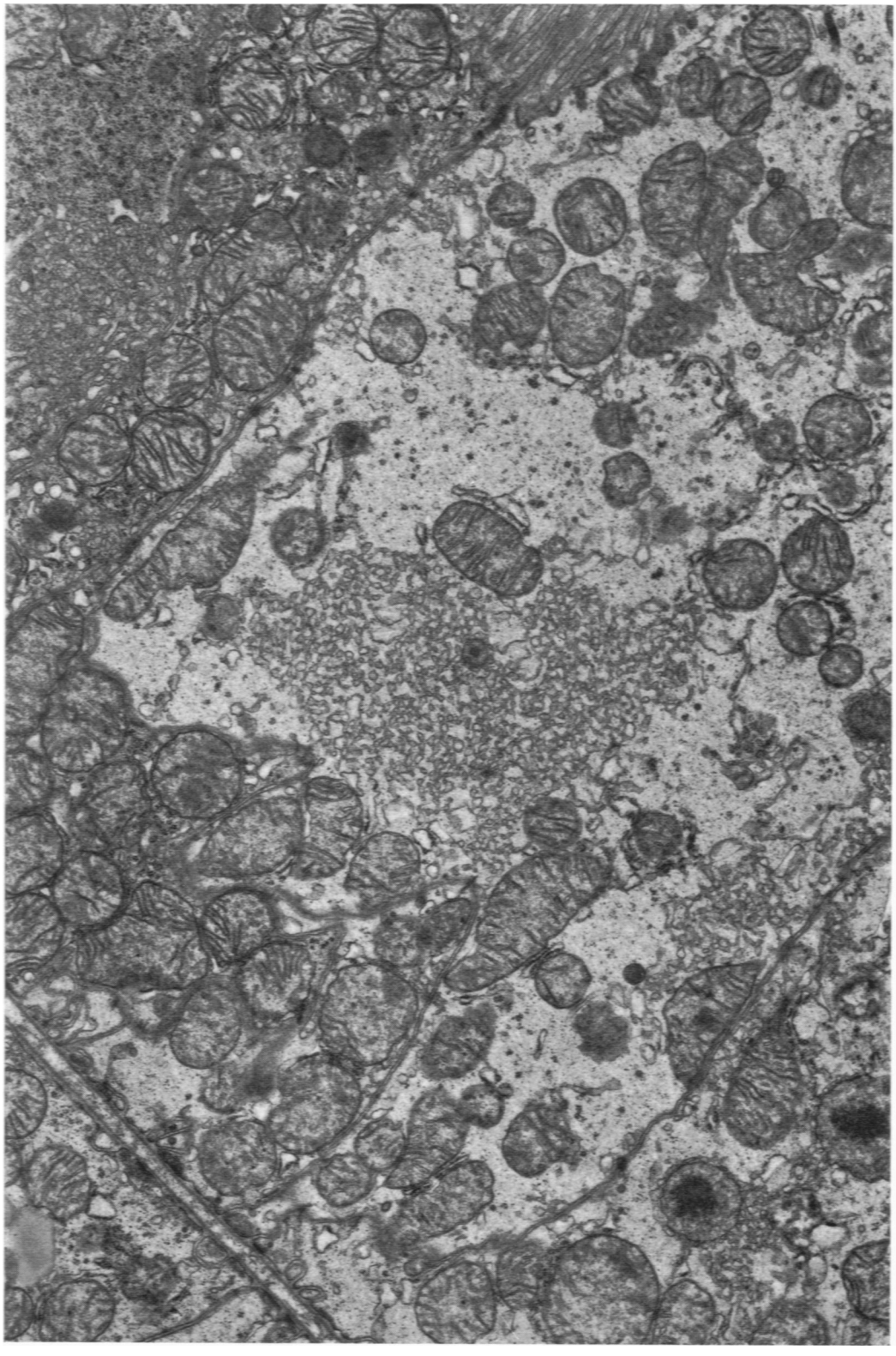

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Fig. 4. Lower midportion of proximal tubule 24 hr. after intoxication. Mitochondria in these cells have resumed a configuration similar to controls. Cell sap is pale. Microvilli are broad, and basilar interdigitations are decreased in number. Large aggregates of smooth-surfaced membranes are numerous. × 16,500.

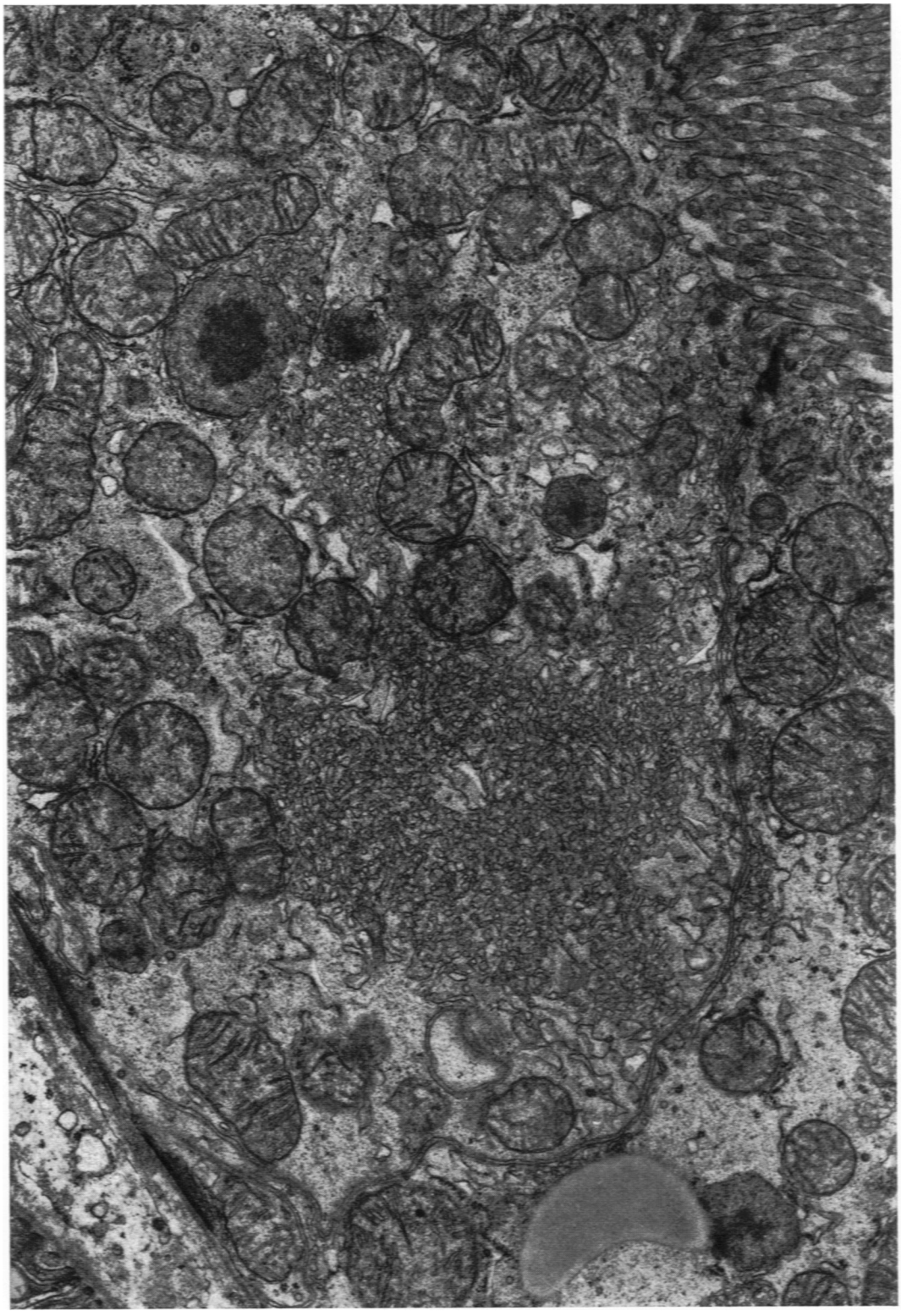

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Fig. 5. Midportion of proximal tubule 24 hr. after intragastric CCl_4. Cellular alterations are similar to those in Fig. 4 and, in addition, show large, single-membrane-bounded vacuoles. × 12,470.

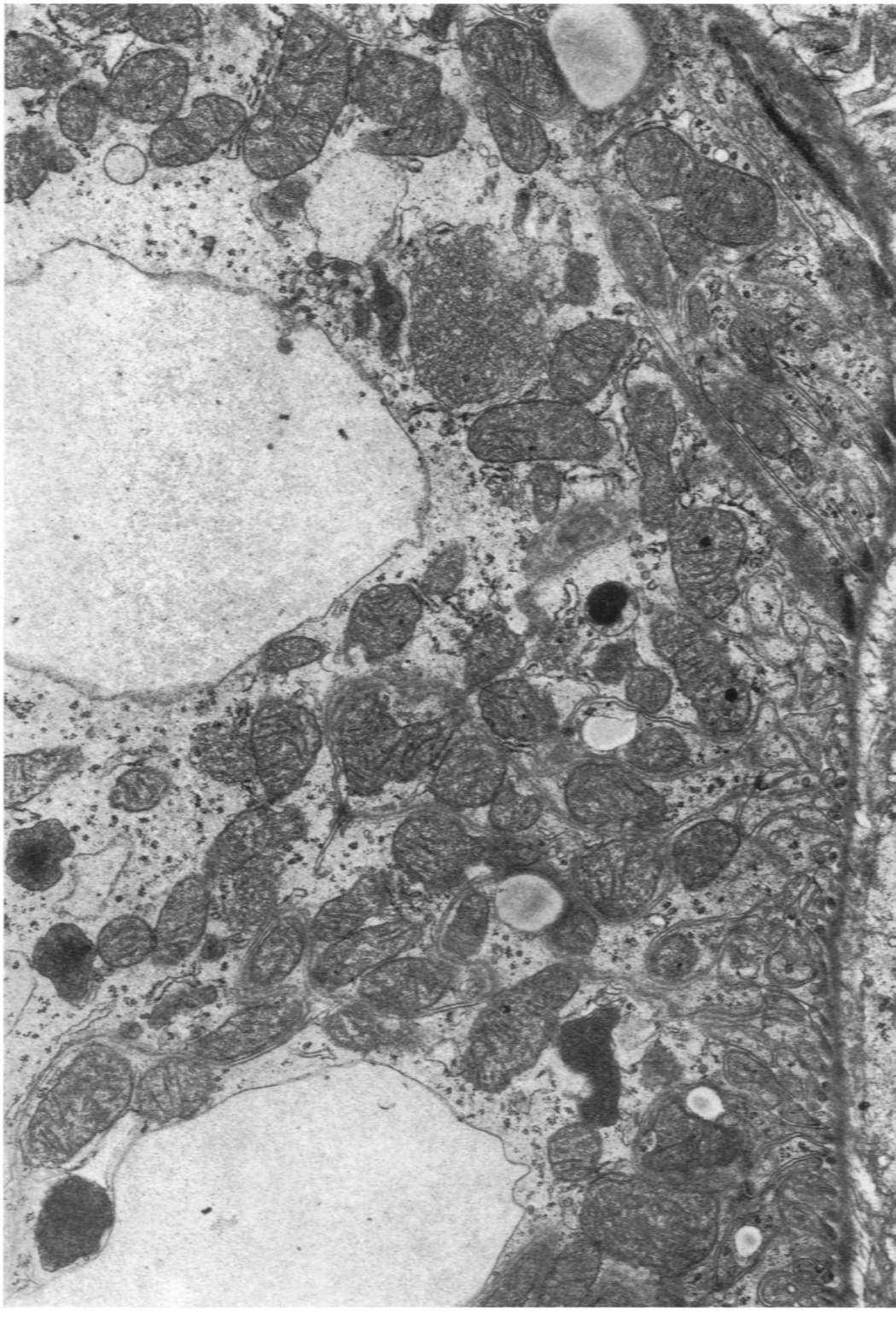

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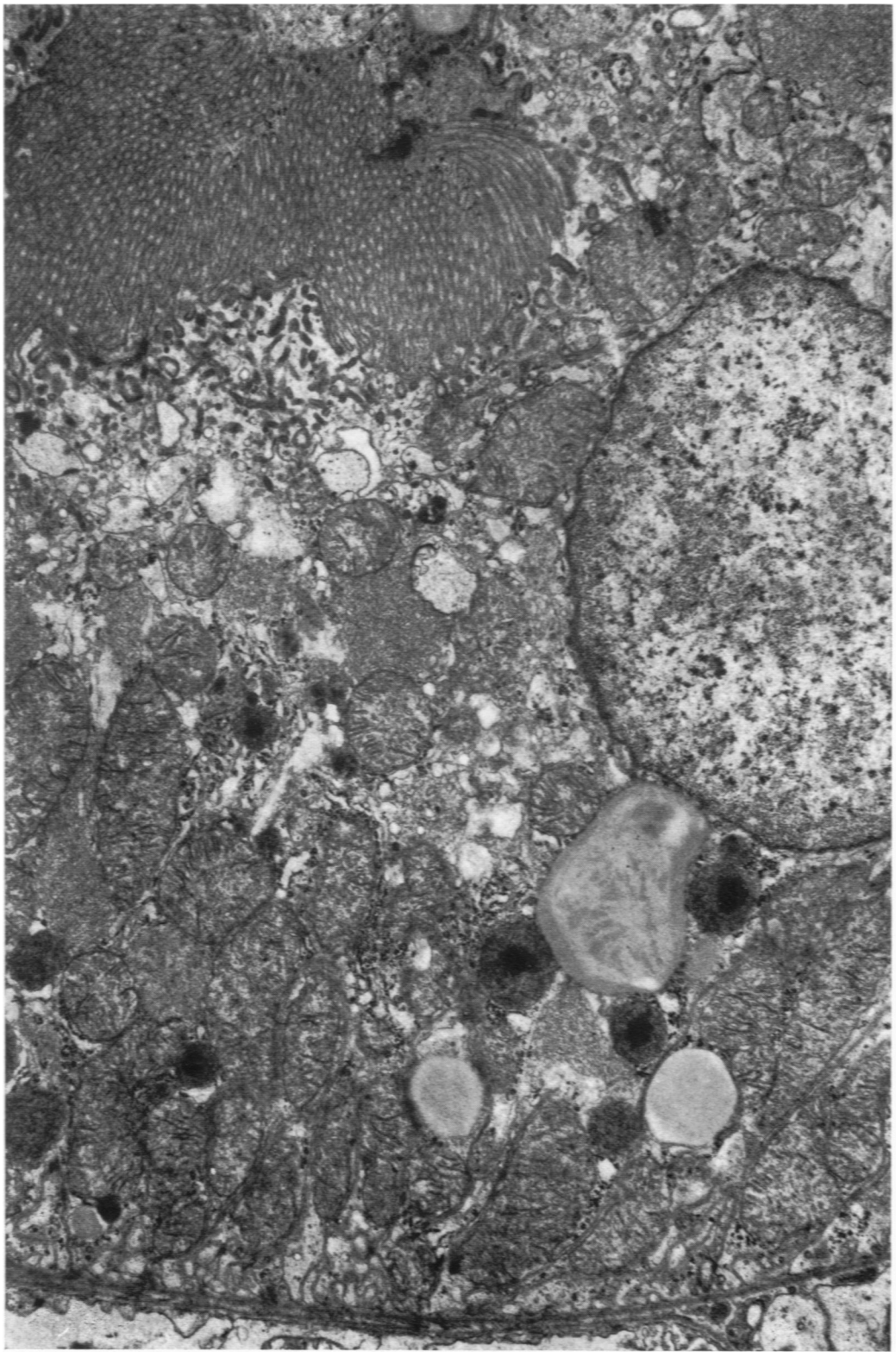
Fig. 6. Midportion of proximal tubule 48 hr. after single dose of CCl_4. Many membranous profiles are scattered throughout the cell. Microbodies and fat droplets are numerous. × 6700.



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