EARLY HEPATIC PARENCHYMAL CHANGES INDUCED IN THE RAT BY AFLATOXIN B_1

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Aflatoxin B_1 has been shown to produce a slowly developing periportal parenchymal cell necrosis following the administration of a single dose.[1] Extractable aflatoxin, however, rapidly disappears from the liver.[2] A maximum is reached at about 1 hour after oral administration, following which there is a rapid disappearance, so that by 6 hours most has gone. By 24 hours when histologic changes are readily identifiable, only a trace is discernible.

In a series of experiments investigating the early biochemical lesion induced by aflatoxin, an inhibition of the liver protein synthetic mechanisms has been described.[3] The disturbance of the protein synthetic mechanisms and that of RNA metabolism were shown to occur rapidly within the first few hours after administration of the toxin. It is the purpose of this paper to describe the early cytologic changes induced by aflatoxin and to attempt to correlate these with the biochemical changes which occur during the same period. The subsequent development of the lesion after 6 hours will be the subject of a further report.

METHODS

Male Wistar rats, 150 gm, were given single administrations of 1 mg aflatoxin B_1. The crystalline toxin (Unilever Ltd., Vlaardingen, The Netherlands) was dissolved in dimethylformamide (DMF) (10 mg aflatoxin B_1 in 1 ml DMF) and given by stomach tube. The animals were not anesthetized for intubation. Control rats were given a similar volume of DMF alone. All animals were treated between 9 A.M. and 10 A.M. and killed 1, 3 and 6 hours later by a blow to the head, 6 dosed and 3 control animals being killed at each time interval. Prior to dosage the rats had free access to M.R.C. diet 41b and water, and after dosage to water alone.

Tissue, taken from the median lobe of the liver, was fixed in 1 per cent OsO_4 in veronal buffer,[4] pH 7.4 for 2 hours at 4° C, dehydrated in graded alcohols and embedded in Epon 812.[5] Ultrathin sections were cut with glass knives on a Huxley Cambridge microtome and stained with 2 per cent uranyl acetate [6] and also in some cases with lead hydroxide.[7] The sections were examined either with an A.E.I. EM6 or a RCA EMU3 microscope. Adjacent thicker sections were stained with either Nile blue sulphate [8] or toluidine blue [9] and examined with the light microscope for orientation.

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Part of the remaining tissue was fixed in 10 per cent formol saline; paraffin sections prepared in the usual fashion were stained with Ehrlich's hematoxylin and eosin.

Results

Controls. The cytoplasmic organelles were essentially similar to those described by other workers.[10,11] Mitochondria were elongated or oval with an even, finely granular matrix and a few electron dense granules. The rough endoplasmic reticulum (RER) appeared as orderly stacks of cisternae or as fewer cisternae passing between mitochondria (Fig. 1). Most ribosomes were attached to the membranes, while some, single or in clusters, lay adjacent to the membranes. In the 3- and 6-hour groups, there was some irregularity of the cisternae with local areas of dilatation. During the course of the experiment, the glycogen areas in the parenchymal cells became less abundant. The Golgi appeared as a series of flattened vesicles. Within the lumen of these cisternae, osmiophilic granules could be seen. Similar granules were observed in the space of Disse.

The parenchymal nuclei were similar in each group and compared with those which have been described.[11,12] The form of nucleolus with the granules arranged as strands (Fig. 2) was more common than that which had a more homogeneous appearance with a prominent perinucleolar rim of chromatin (Fig. 3).

Experimental—1 Hour. Parenchymal cells in the centrilobular zone were normal. In the periportal region some cells showed dilatation of the cisternae of RER with a few dislocated ribosomes (Fig. 4). This was the only change seen at this time. The other cytoplasmic organelles and nuclei within the altered cell exhibited a normal appearance. The remainder of the periportal cells were similar to those in the controls.

3 Hours. The change in the periportal cells at 1 hour was now much more evident, with all the cells in the zone being affected. The stacks of RER showed greater dilation and cisternal irregularity (Fig. 5). In some areas, ribosomes were distributed at random in the inter-cisternal space and comparatively long segments of membrane were free of ribosomes. Within the dilated cisternal spaces were round granules of varying density. The Golgi was also dilated and contained similar granules within the lumens. The SER showed no significant change. The mitochondria maintained regular outlines with even distribution of matrix and electron dense granules.

The centrilobular cells at this stage showed no change from the controls (Fig. 6). The stacks of RER were regular and there was no dilatation of the cisternae. The ribosomes were associated with membranes of the ER and the SER and cytoplasmic organelles were normal.

Nuclear alterations did not exhibit the zonal distribution characterizing the cytoplasmic changes. In the control material and at 1 hour after treatment the nucleolar configuration with prominent perinucleolar chromatin (Fig. 3) was less evident than that illustrated in Figure 2. At 3 hours, however, nucleoli with prominent perinucleolar chromatin and indistinct nucleolemma were abundant. Some of the nucleoli (Fig. 9) appeared to be shrunken and dense and exhibited zoning of the nucleolar substance. A varying number of zones could be distinguished but usually two were readily apparent. Zone A appeared as a coarsely granular area containing ribosome-like particles and zone B exhibited a more homogeneous appearance. This type of nuclear change was noted in cells with grossly abnormal cytoplasm in the periportal zone, as well as in the centrilobular area in which there was minimal or no change in the RER.

6 Hours. The periportal zone now exhibited complete disruption of the stacks of RER with the formation of irregular vesicles (Fig. 7). Many free ribosomes were scattered throughout the cytoplasm either singly or in clusters. The Golgi now showed greater irregularity and dilatation of its cisternae. Within the intracisternal spaces in both the Golgi and the remnants of RER dense granules were encountered as in the earlier groups and the controls. The SER at this stage consisted of irregular vesicles (Fig. 7) and was not readily distinguished from the disrupted RER.

The mitochondria now exhibited irregular profiles. Blebs and infoldings of the membranes consisted of either the outer or the inner membrane, or comprised a double membrane. In some instances, there appeared to be a discontinuity of the inner membrane (Fig. 8). The granular matrix was rather patchy and only occasional electron dense granules were noted within the mitochondria. At this stage cytoplasmic organelles appeared unchanged. A few small fat droplets could be seen (Fig. 8) but at no stage up to 6 hours was the accumulation of fat a prominent feature.

Some centrilobular cells were beginning to show alterations (Fig. 10). Although regular stacks of RER, with associated ribosomes, remained in some areas, there was dilatation of cisternae and dislocation of ribosomes. The mitochondria showed some loss of matrix but did not have the irregular outline seen in the periportal zone.

Changes within the nucleolus seen at 3 hours were still present in this group but appeared to be neither more abundant nor with more of a zonal distribution.

In no instance at any stage was there any abnormality of the cell membrane, either abutting upon the adjacent parenchymal cells or the space of Disse.

Discussion

DMF has been selected as a suitable solvent since, at the dose used, no evidence of pathologic alterations was seen in the original experiments describing the acute toxicity of aflatoxin B_1.[1] This was confirmed by biochemical studies[8] and in the present investigation no ultrastructural change appeared in the controls which could be attributed to DMF. The only difference from the control groups was a slight dilatation of the RER cisternae at 3 and 6 hours. Similar changes have been reported by other workers.[13–15]

The earliest change seen in this study was in the RER. As early as 1 hour, a few periportal parenchymal cells showed dilatation of RER cisternae and dislocation of ribosomes. This was progressive so that by 3 hours, all the cells in the periportal zone were affected and the degree of disruption was greater. At 6 hours, the change was even more marked with complete disruption of the stacks of RER and free ribosomes scattered throughout the cytoplasm. At this stage, alteration of mitochondrial and nucleolar structure was also noted and centrilobular cells exhibited some dislocation of ribosomes and dilatation of cisternae.

As mentioned previously, aflatoxin B_1 induces a periportal zone of necrosis. This can be recognized by 20 to 24 hours in paraffin embedded tissue stained with hematoxylin and eosin and has been described in detail in a previous paper.[1] It was also shown that with the dosage used no histologic evidence of necrosis was detected in centrilobular cells. This correlated well with the present observation that the most severely affected cells were those in the periportal region. It is not possible to say, however, whether the cytoplasmic disruption in these cells was sufficient in itself to lead to necrosis. In the centrilobular area, there was some change in the RER by 6 hours but necrosis did not develop subsequently. It is not known how far the dilatation of RER cisternae and dislocation of ribosomes might progress in the centrilobular zone.

It has been demonstrated that in normal liver parenchymal cells, protein synthetic mechanisms are associated with the RER.[16,17] It has been suggested that the electron dense granules of the RER and Golgi cisternae are secretory granules of protein or lipid.[11,18]

Within 1 hour of administration of carbon tetrachloride (CCl_4), dilatation of the RER cisternae has been demonstrated.[19] By 3 hours, dislocation of ribosomes has been described[15] and the liver subsequently becomes fatty. A failure of C_{14} amino acids to incorporate into proteins has also been shown to exist at these times.[15] Triglyceride is transported in the form of lipoprotein, thus a failure to form the protein moiety might be expected to result in a failure of triglyceride transport. Similarly

following dosage with dimethylnitrosamine (DMN), there is a correlation between the disaggregation of the RER, fat accumulation and the inhibition of protein synthesis.[14,20,21] Both of these toxins produce a centrilobular lesion.

Aflatoxin B_1 has been shown to inhibit incorporation of C_{14} amino acids into proteins and to inhibit induction of new enzymes as early as 1 hour after administration.[3,22,23] The early structural changes induced by aflatoxin, although periportal in distribution, are similar to those seen with both DMN and CCl_4, except that there is no significant fatty change. This may reflect either the zonal nature of the lesion, or intimate that lipoprotein synthesis is not affected to such a degree. The latter suggestion is further supported by the observation that the intracisternal electron dense granules can be still seen at 3 and 6 hours after aflatoxin administration. In spite of the fact that the earliest changes occurred at the same time as those induced by DMN or CCl_4, necrosis develops much later, so that a well developed zonal necrosis is not seen until 48 hours.

Another feature of note was the nucleolar alteration observed at 3 and 6 hours. The zoning of the nucleoli was similar to the development of nucleolar caps reported in tissue cultures treated with either actinomycin D or mitomycin C.[24,25]

Actinomycin D has been shown to inhibit the synthesis of DNA dependent RNA and to have an effect on protein synthesis.[26–28] Smuckler and Benditt[27] have described altered nucleoli in the rat liver after the administration of actinomycin D but failed to find any alteration of the RER. Similarly Jézéquel and Bernhard[28] have described the formation of nucleolar caps in the rat pancreas but failed to find any alterations of the RER or zymogen granules. Aflatoxin has also been shown to inhibit RNA synthesis[29,30] and when given after partial hepatectomy Bernhard, Frayssinet, Lafarge and LeBreton[31] demonstrated nucleolar alterations but no cytoplasmic change. In the present study in which profound cytoplasmic changes occurred, there appeared to be no correlation between the nucleolar and cytoplasmic alterations. This would suggest that the cytoplasmic change was independent of the nucleolar alterations.

Summary

Sequential ultrastructural changes were induced in rat liver parenchymal cells by the oral administration of aflatoxin B_1. These were described at intervals of 1, 3 and 6 hours.

At 1 hour some dilatation of the RER cisternae and dislocation of ribosomes appeared in periportal cells. By 3 hours disruption of the RER was more evident; centrilobular cells remained normal. At 6

hours when the periportal cells exhibited further disruption with alteration in mitochondria, centrilobular parenchymal cells revealed dilatation of RER cisternae and dislocation of ribosomes. At 3 and 6 hours the formation of nucleolar caps was a feature.

There appears to be a correlation between inhibition of the protein synthetic mechanism and the disruption of the RER but this does not appear to be related to the nucleolar alterations.

References

1. Butler, W. H. Acute toxicity of aflatoxin B_1 in Rats. *Brit. J. Cancer,* 1964, 18, 756–762.
2. Butler, W. H., and Clifford, J. I. Extraction of aflatoxin from rat liver. *Nature (London)*, 1965, **206**, 1045–1046.
3. Clifford, J. I., and Rees, K. R. Aflatoxin: a site of action in the rat liver cell. *Nature (London)*, 1966, **209**, 312–313.
4. Palade, G. E. A study of fixation for electron microscopy. *J. Exp. Med.,* 1952, 95, 285–298.
5. Luft, J. H. Improvements in epoxy resin embedding methods. *J. Biophys. & Biochem. Cytol.,* 1961, 9, 409–414.
6. Watson, M. L. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. & Biochem. Cytol.,* 1958, 4, 475–478.
7. Millonig, G. A modified procedure for lead staining of thin sections. *J. Biophys. & Biochem. Cytol.,* 1961, 11, 736–739.
8. McGee-Russell, S. M., and Smale, N. B. On colouring Epon-embedded tissue sections with Sudan black B or Nile blue A for light microscopy. *Quart. J. Micr. Sci.,* 1963, 104, 109–115.
9. Trump, B. F.; Smuckler, E. A., and Benditt, E. P. A method for staining epoxy sections for light microscopy. *J. Ultrastruct. Res.,* 1961, 5, 343–348.
10. Fawcett, D. W. Observations on the cytology and electron microscopy of hepatic cells. *J. Nat. Cancer Inst.,* 1955, 15 (suppl.), 1475–1503.
11. Bruni, C., and Porter, K. R. The fine structure of the parenchymal cell of the normal rat liver. I. General observations. *Amer. J. Path.,* 1965, 46, 691–755.
12. Davis, J.M.G. The Ultrastructure of the Mammalian Nucleolus. In: The Cell Nucleus. Mitchell, J. S. (ed.). Academic Press, New York, 1960, pp. 3–14.
13. Herdson, P. B.; Garvin, P. J., and Jennings, R. B. Fine structural changes produced in rat liver by partial starvation. *Amer. J. Path.,* 1964, 45, 157–181.
14. Gustafsson, R. G., and Afzelius, B. A. Comparative effects on rat liver cells after dimethylnitrosamine, 2.-fluorenamine, or prednisolone treatment studied by electron microscopy. *J. Nat. Cancer Inst.,* 1963, 30, 1045–1075.
15. Smuckler, E. A.; Iseri, O. A., and Benditt, E. P. An intracellular defect in protein synthesis induced by carbon tetrachloride. *J. Exp. Med.,* 1962, 116, 55–72.
16. Palade, G. E., and Siekevitz, P. Liver microsomes; an integrated morphological and biochemical study. *J. Biophys. & Biochem. Cytol.,* 1956, 2, 171–200.
17. Siekevitz, P., and Palade, G. E. A cytochemical study on the pancreas of the guinea pig. VI. Release of enzymes and ribonucleic acid from ribonucleoprotein particles. *J. Biophys. & Biochem. Cytol.,* 1960, 7, 631–644.

18. TROTTER, N. L. A fine structure study of lipid in mouse liver regenerating after partial hepatectomy. *J. Cell Biol.*, 1964, **21**, 233–244.

19. BASSI, M. Electron microscopy of rat liver after carbon tetrachloride poisoning. *Exp. Cell. Res.*, 1960, **20**, 313–323.

20. EMMELOT, P., and BENEDETTI, E. L. Changes in the fine structure of rat liver cells brought about by dimethylnitrosamine. *J. Cell Biol.*, 1960, **7**, 393–396.

21. MAGEE, P. N. Toxic liver injury. Inhibition of protein synthesis in rat liver by dimethylnitrosamine *in vivo*. *Biochem. J.*, 1958, **70**, 606–611.

22. SHANK, R. C., and WOGAN, G. N. Effects of aflatoxin B_1 on some aspects of liver metabolism. (Abstract) *Fed. Proc.*, 1964, **23**, 200.

23. WOGAN, G. N., and FREIDMAN, M. A. Effects of aflatoxin B_1 on tryptophan pyrrolase induction in rat liver. (Abstract) *Fed. Proc.*, 1965, **24**, 627.

24. REYNOLDS, R. C.; MONTGOMERY, P. O'B., and HUGHES, B. Nucleolar 'caps' produced by actinomycin D. *Cancer Res.*, 1964, **24**, 1269–1277.

25. LAPIS, K., and BERNHARD, W. The effect of mitomycin-C on the nucleolar fine structure of KB cells in cell culture. *Cancer Res.*, 1965, **25**, 628–645.

26. REICH, E. Biochemistry of actinomycins. *Cancer Res.*, 1963, **23**, 1428–1441.

27. SMUCKLER, E. A., and BENDITT, E. P. The early effects of actinomycin on rat liver. Changes in ribosomes and polysomes. *Lab. Invest.*, 1965, **14**, 1699–1709.

28. JÉZÉQUEL, A-M., and BERNHARD, W. Modifications ultrastructurales du pancréas exocrine de rat sous l'effet de l'actinomycine D. *J. Microscopie*, 1964, **3**, 279–296.

29. LAFARGE, C.; FRAYSSINET, C., and DERECONDO, A. M. Inhibition par l'aflatoxine de la synthèse de RNA hépatique chez le rat. *Bull. Soc. Chim. Biol.*, 1965, **47**, 1724–1725.

30. FREIDMAN, M. A., and WOGAN, G. N. Effects of aflatoxin B_1 on enzyme induction and nuclear RNA metabolism in the rat. (Abstract) *Fed. Proc.*, 1966, **25**, 662.

31. BERNHARD, W.; FRAYSSINET, C.; LAFARGE, C., and LEBRETON, E. Lésions nucléolaires précoces provoquées par l'aflatoxine dans les cellules hépatiques du rat. *C. R. Acad. Sci. (Paris)*, 1965, **261**, 1785–1788.

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[*Illustrations follow*]

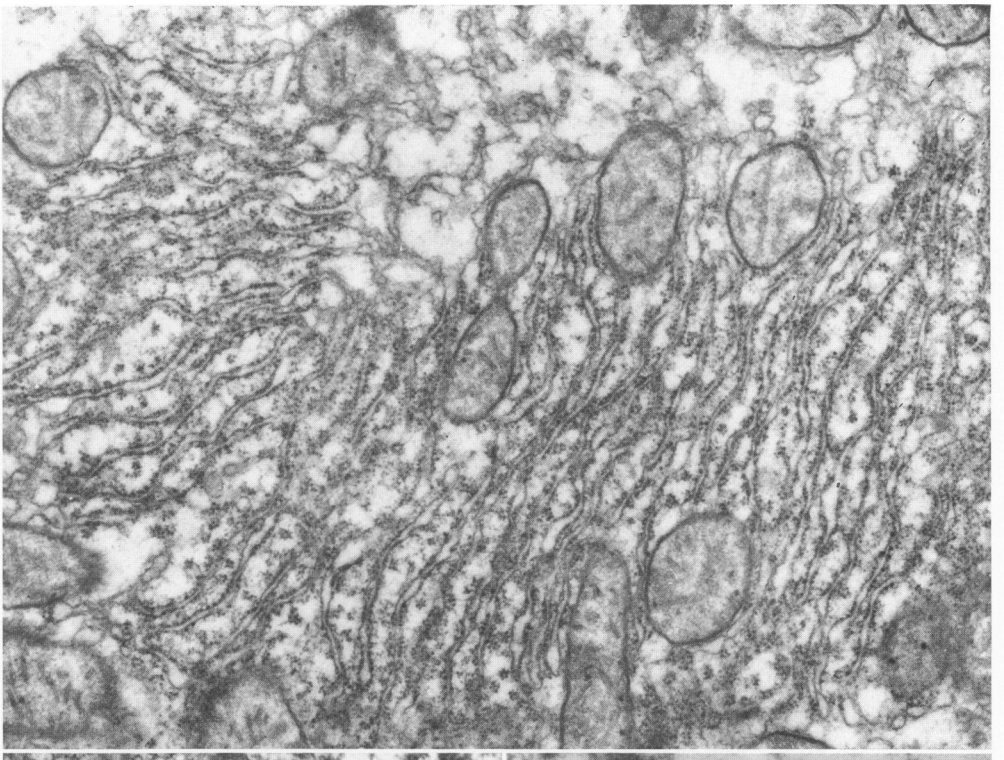
Legends for Figures

Unless otherwise indicated, micrographs were prepared from sections stained with uranyl acetate.

Fig. 1. Part of a liver parenchymal cell, 3-hour control. Stacks of RER with associated ribosomes. × 24,000.

Fig. 2. Nucleolus of a liver parenchymal cell, 3-hour control. The nucleolus consists mainly of dense ribosome-like particles and a loose network of the nucleolenema. × 22,600.

Fig. 3. Nucleolus of a liver parenchymal cell, 3-hour control rat. A uniformly dense finely granular material exhibits areas of low density and a prominent perinucleolar rim of chromatin. × 22,800.



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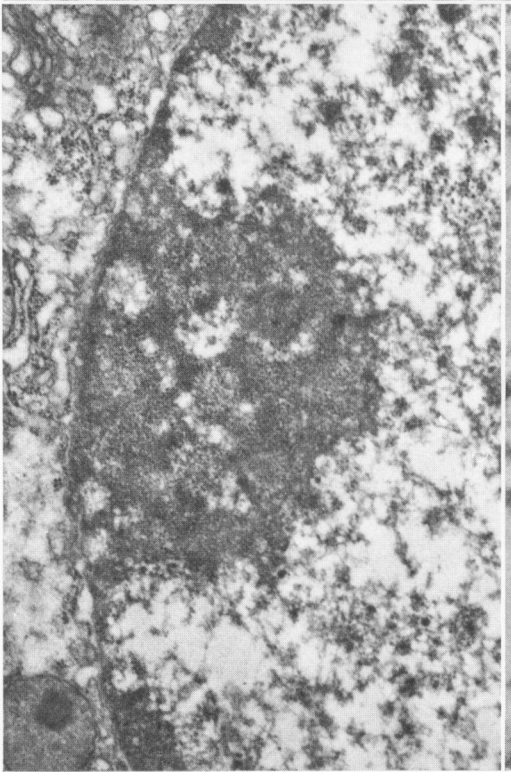

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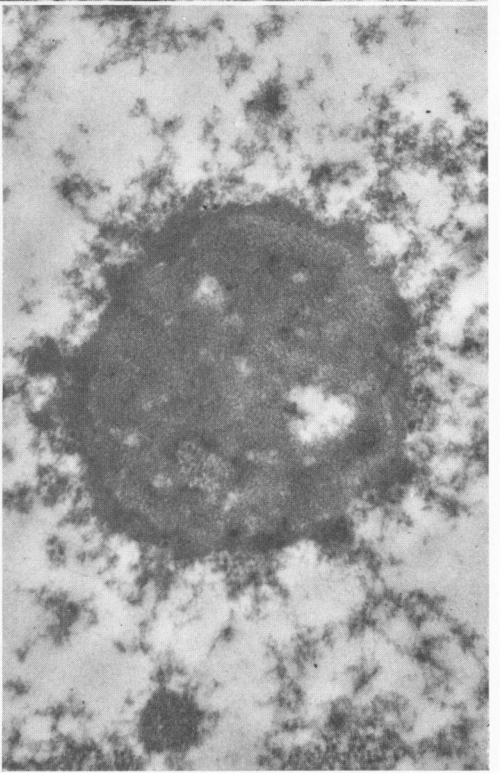

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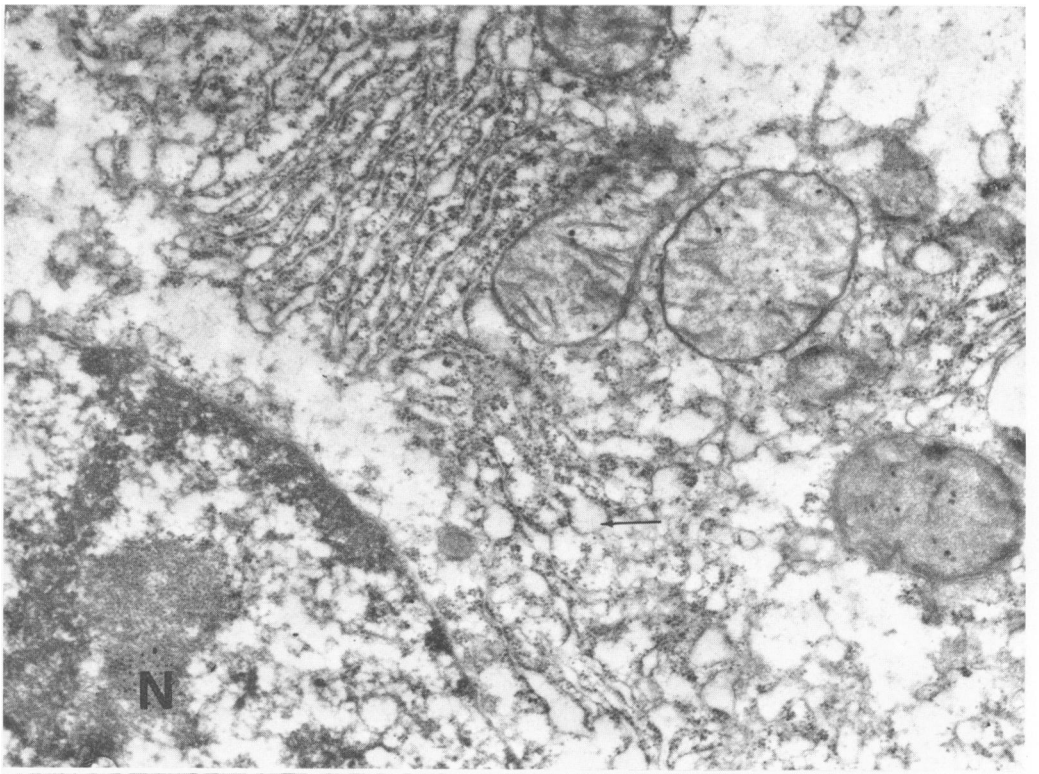
FIG. 4. Part of the nucleus (N) and the cytoplasm of a periportal parenchymal cell, 1 hour after administration of aflatoxin B_1. The RER shows some dilatation of cisternae (arrow) and dislocation or ribosomes. × 22,700.

FIG. 5. Part of a nucleus (N) and the cytoplasm of a periportal parenchymal cell, 3 hours after administration of aflatoxin B_1. The RER cisternae are irregularly dilated with the formation of vesicles (arrow). Free ribosomes are present in the intercisternal spaces. Dense granules can be seen within the dilated Golgi (g) and within vesicles of RER (r). × 17,100.



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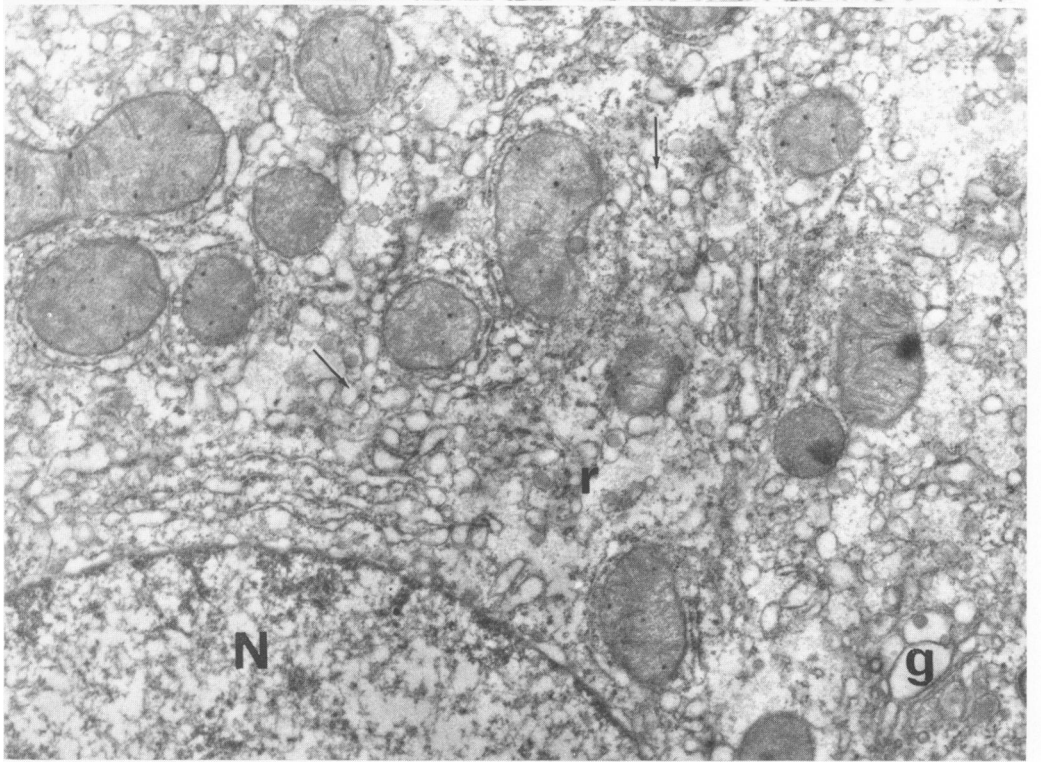

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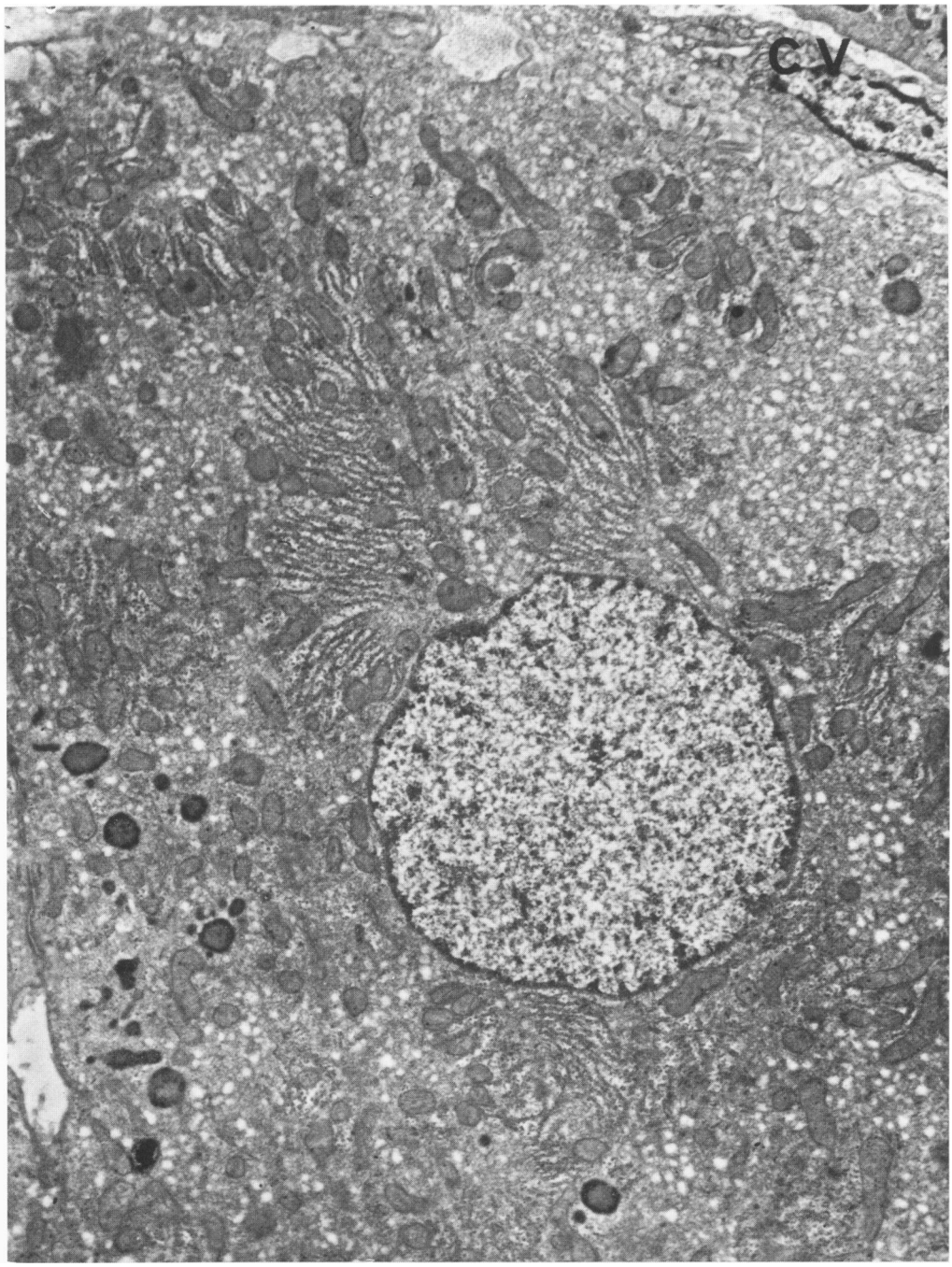

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FIG. 6. A centrilobular parenchymal cell from the rat liver illustrated in Figure 5. The stacks of RER maintain a normal arrangement. Central vein (CV). × 8,500.

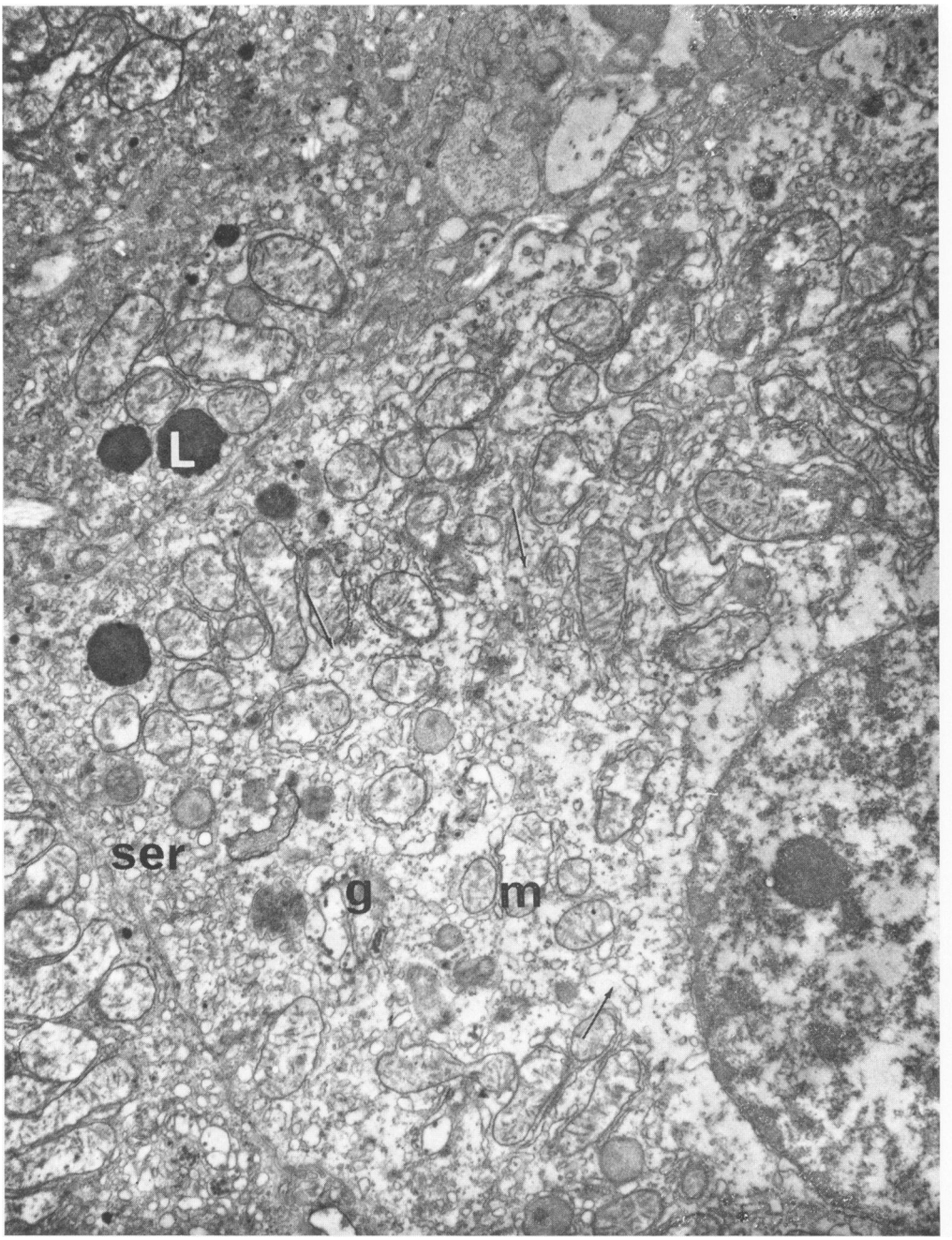
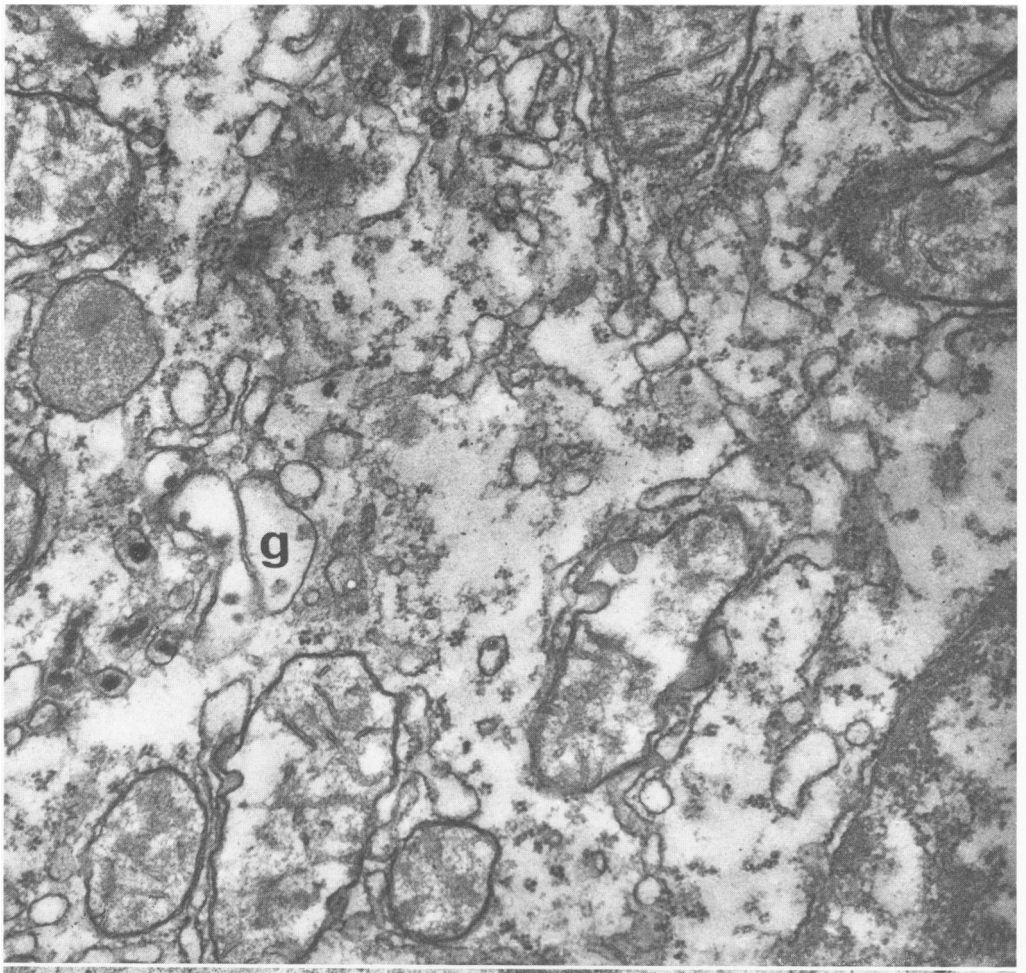


FIG. 7. Part of a periportal parenchymal cell, rat killed 6 hours after treatment with aflatoxin. The RER consists of irregular vesicles (arrows); the SER has a similar appearance (SER). Mitochondria (M) have irregular profiles. The Golgi (g) is dilated and contains dense secretory granules. Lipid (L). Uranyl acetate and lead hydroxide stain. × 10,500.

FIG. 8. The same field shown in Figure 7. Irregular vesicles of RER and dilated Golgi (g) with dense secretory granules are evident. Mitochondria exhibit a loss of matrix and irregular profiles of membranes. Clusters of ribosomes are scattered throughout the field. Uranyl acetate and lead hydroxide stain. × 40,000.

FIG. 9. Nucleolus of a parenchymal cell, rat killed 6 hours after treatment with aflatoxin. The nucleolar material exhibits zoning. Zone A consists of ribosome-like particles, zone B is more homogeneous. × 29,200.



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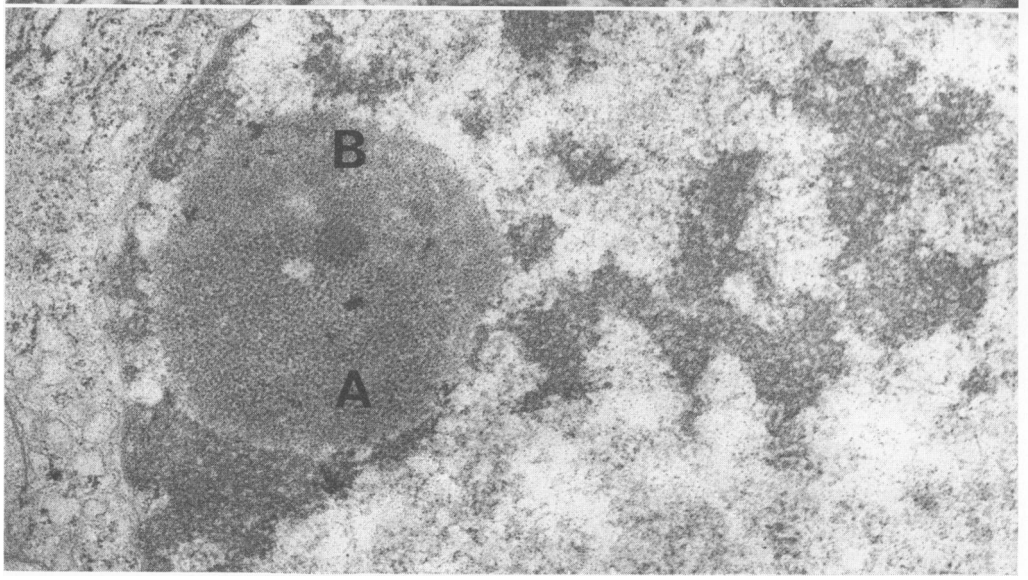

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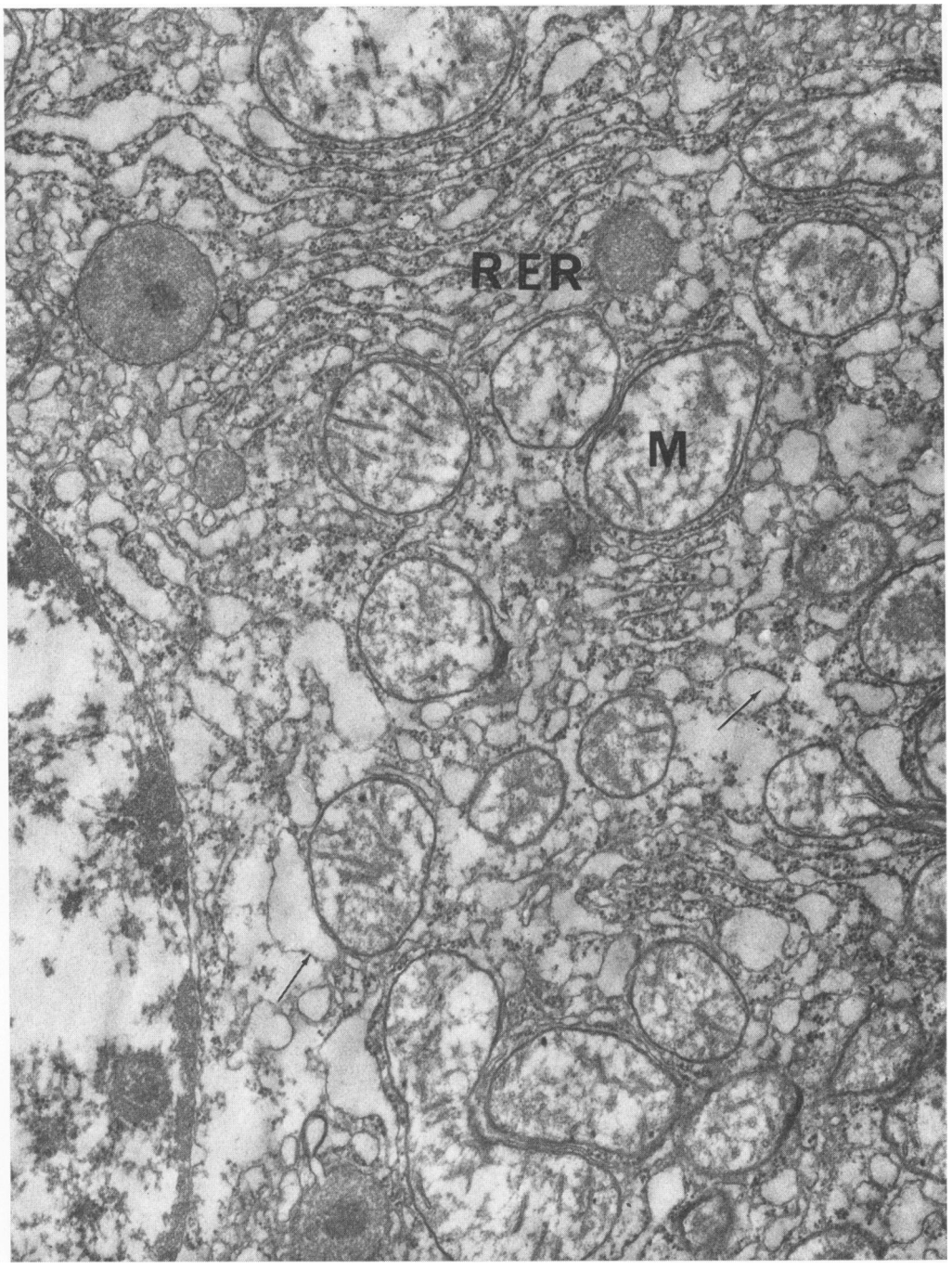

FIG. 10. A centrilobular parenchymal cell, rat killed 6 hours after treatment with aflatoxin. An irregular dilatation of RER cisternae is accompanied by free ribosomes (arrow). Other areas show orderly stacks of RER. Mitochondria (M) are swollen and exhibit some loss of matrix. × 28,000.