AFLATOXIN B_1 INJURY IN RAT AND MONKEY LIVER

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Aflatoxin, a substituted lactone, is a metabolite of certain strains of *Aspergillus flavus*,[1] a common mould which has been isolated from many foodstuffs, including peanuts, wheat, rice, soybean, corn, bread, milk and cheese,[2] especially when stored in hot, moist conditions.[1] The fungus also has been found in tropical soils. The toxic principle was first recognized in Great Britain [3] where it caused fatal liver disease in turkeys, and it was later shown to be an extremely potent carcinogen.[4-6] The various chemical forms of aflatoxin have since been purified and identified.[7-9]

Because of its worldwide distribution, and especially its prominent occurrence in Africa, where circumstantial evidence has suggested that foods may be contaminated by mycotoxins,[2,10] the potential significance of aflatoxin in human cancer has aroused concern.

As part of a systematic study designed to delineate and compare the acute, reversible subcellular changes produced by hepatic carcinogens with those that predominate and persist during the precancerous stages, we have examined some of the effects of aflatoxin. We were also interested in determining whether this toxin produces specific acute and chronic hepatic lesions in animals. This would assist in elucidating the hypothetical role of the toxin in man.

MATERIAL AND METHODS

The aflatoxin B_1 used in these experiments was obtained through the generous cooperation of Dr. G. N. Wogan (Massachusetts Institute of Technology). A "crude" aflatoxin B_1 used in some of the earlier experiments was stated by Dr. Wogan to be approximately one-fourth as strong as the pure preparation. The lesions produced by both preparations, however, were qualitatively similar. The agent was administered as a solution in ethanol or in ethanol/propylene glycol but the vehicle and the route of administration did not affect the qualitative nature of the lesions.

Acute Studies

Rats. Inbred male F-344 rats (250 to 300 gm) were used. Rats whose tissues were to be used for electron microscopic studies were divided according to the dose schedules in Table I. They were fed Purina® Chow, but were starved 24 hours before sacrifice.

Monkeys. Six female rhesus monkeys weighing approximately 2 kg were treated as follows:

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(1) 3 mg per kg, pure aflatoxin, by mouth (PO) (died at 96 hours),
(2) 2.6 mg per kg, pure, intraperitoneally (IP) (sacrificed at 24 hours),
(3) 2.6 mg per kg, pure, IP (died at 60 hours),
(4) 2.5 mg per kg, pure, IP (sacrificed at 48 hours),
(5) 0.45 mg per kg, pure, PO (sacrificed at 48 hours),
(6) 2.6 mg per kg, crude, IP (sacrificed at 48 hours).

Electron microscopic studies were carried out in animals 2, 4, 5 and 6. Light microscopy was accomplished on the tissues of all monkeys.

Chronic Studies

Sixteen male weanling F-344 rats were fed pure aflatoxin B_1, 1 part per million (ppm) in a synthetic diet *ad libitum* (16 per cent vitamin-free casein; 10 per cent corn oil; 62.5 per cent sucrose; 2.5 per cent vitamin mix; 4 per cent salt mixture, USP XIV; 5 per cent Alphacel). After 16 weeks, in 9 animals, the dose was increased to 2 ppm. At 33 weeks, all animals were placed on Purina® Chow for an additional 19 weeks. During the entire experiment, laparotomy was performed and biopsy made as indicated in Table II. Laparotomies were performed on all animals at 33 weeks and complete necropsies were done at sacrifice.

TABLE I

DOSE SCHEDULES FOR ACUTE TOXICITY EXPERIMENTS IN RATS

Group	Number of rats *	Dose	Route	Sacrifice
		Pure aflatoxin B_1		
1	2	3.78 mg/kg	PO	72 hours
2	4	1.89 mg/kg	PO	24, 48 & 72 hours
3	12	0.45 mg/kg	PO	24, 48 & 72 hours
4	6	0.167 mg/kg	IP	24, 48 & 72 hours
		Crude aflatoxin B_1		
5	7	2.6 mg/kg	IP	24, 48 & 72 hours
6	2	2.0 mg/kg	IP	1 hour
7	8	1.0 mg/kg	IP	12, 24, 48 & 72 hours

* This refers only to those rats used for the electron microscopic study. Additional rats were used for light microscopy and for biochemical analysis.

TABLE II

CHRONIC AFLATOXIN EXPERIMENTS IN RATS

Group	No. animals	Aflatoxin concentration in diet	Duration	Biopsy (wks.)	Appearance of first tumor	Animals with tumors
1	6	1 ppm	33 weeks	2, 6, 10, 12, 14, 18, 20, 24, 35, 37, 39, 40, 52	35 weeks	3/6
2	9	1 ppm followed by 2 ppm	16 weeks 17 weeks	18, 20, 22, 26, 28, 30 35, 37, 39, 40, 48, 52	26 weeks	9/9

Morphologic Studies

Electron Microscopy. Each rat was lightly anesthetized with Metofane and its abdomen opened by a midline incision. Slices of liver were removed from either the median or lateral lobes, including tumor if present; these were minced into cubes measuring 1 mm or less and fixed for 1 hour in 2 per cent osmium tetroxide buffered to pH 7.4 with *s*-collidine. Additional samples were fixed in phosphate-buffered glutaraldehyde. The tissue was dehydrated in a graded series of alcohols and embedded in Epon 812. Thin sections cut with an LKB Ultratome were mounted on titanium grids, stained with lead or uranium and examined with an RCA 3G electron microscope employing a 35- to 40-μ objective aperture and an accelerating voltage of 50 kv.

Samples of pancreas were taken from 2 animals at each interval in group 3 (Table I).

The monkeys were anesthetized with Nembutal® (30 mg per kg IP) and samples of liver quickly removed. The tissue was subsequently prepared for electron microscopy with the same methods used for rat liver.

Light Microscopy. Samples of all viscera from both rats and monkeys were fixed in neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. Selected blocks from both species were stained with the periodic acid-Schiff (PAS) technique, the Feulgen stain and methyl green–pyronine. Frozen sections were stained with oil red O for fat. Semithin (0.5 to 1.5 μ) sections of liver prepared for electron microscopy were stained with toluidine blue for light microscopic examination.

Biochemical Studies

Specimens of rat liver were homogenized in a Potter-Elvehjem homogenizer and the nuclear fraction separated by the method of Widnell and Tata.[11] Purity of the fraction was confirmed by electron microscopic examination of embedded pellets and by estimation of the glucose-6-phosphatase activity in the fraction.[12,13] Nucleic acids were determined by the method of Schneider.[14] Protein was estimated by the Lowry method.[15] For total lipids, liver was dried to a constant weight, extracted with Bloor's ethanol–ether solvent, reweighed and the lipid content determined by difference. Glycogen was determined by the method of Seifter [16] using the anthrone reagent. These biochemical determinations were performed in duplicate and the results expressed per mg DNA.

Phosphorous–oxygen (P:O) ratios were determined with alphaketoglutarate as the substrate as outlined by Umbreit, Burris and Stauffer.[17] Malonate was included to block oxidation of succinate.

RESULTS

Acute Experiments

Rats. Light Microscopy. In our experience a dose of 0.45 mg per kg as used in group 3 was the most suitable to permit study of cytologic changes without excessive necrosis during the period of the acute experiments. Accordingly, the following descriptions related to both light and electron microscopy are concerned with this group except where otherwise indicated.

In groups 2 and 3, at 24 hours there was slight swelling of periportal parenchymal cells and increased acidophilia; there was also loss of glycogen and pyroninophilia in the periportal and midzonal areas. Scanty mononuclear cell infiltrates were present about some of the portal tracts.

By 48 to 72 hours, there was well marked necrosis of periportal liver cells with a neutrophil and mononuclear cell infiltrate. There was slight ductular and bile duct proliferation, some cells showing mitotic figures. Occasional acidophilic bodies were seen in liver and Kupffer cells. At these intervals nucleoli stained with Feulgen stain, methyl green–pyronine and hematoxylin and eosin, were much smaller and less numerous than in the normal state. At 72 hours necrosis was more apparent. In 2 rats given 2 mg per kg crude aflatoxin and sacrificed at 1 hour (group 6) no light microscopic changes were detected; by electron microscopy, however, nuclear alterations (see below) were conspicuous. In rats given 0.167 mg per kg pure aflatoxin IP (group 4) no necrosis was observed and changes in periportal parenchymal cells were negligible.

Electron Microscopy. Twenty-four Hours. The most prominent changes occurred in nuclei; there were diminution in nucleolar size, separation of the fibrillar and granular components of the nucleolus and formation of nucleolar "caps" (Fig. 1). In some cells, complete separation of the fibrillar and granular elements was seen (Fig. 2) while, in other nuclei, nucleolar components were accompanied by dispersed nucleolar granules (Fig. 3). While the lobular orientation in sections examined by electron microscopy was uncertain, examination of semithin sections by light microscopy indicated that the nuclear alterations were present throughout the lobule and were present in almost all cells.

In the cytoplasm there was proliferation of smooth endoplasmic reticulum (Fig. 4) and early focal cytoplasmic necrosis. Dissociation of ribosomes from ergastoplasmic membranes was conspicuous (Fig. 5). In the periportal regions, mitochondria were closely packed and lacked normal matrix dense granules but contained numerous ill-defined flecks of dense material (Figs. 6 and 7).

Forty-eight Hours. By 48 hours, nuclear changes described previously persisted and were of somewhat greater variety although it was not possible to reconstruct a temporal sequence based on their severity. The mitochondria in many cells contained roughly circular areas consisting of a granular interior surrounded by a dense periphery (Fig. 8). In the cytoplasm, extensive myelin figures were common (Fig. 9) and numerous lipid droplets were present within cisterns of endoplasmic reticulum (Fig. 10). Dissociation of ribosomes from ergastoplasmic membranes persisted.

Seventy-two Hours. Nuclear abnormalities persisted and small condensations of the fibrillar component measured approximately 0.5 to 0.7 μ in diameter. This component was frequently closely associated with a collection of dense granules.

Aside from necrosis of periportal cells, the cytoplasmic changes at 72

hours consisted of enhanced proliferation and dilatation of smooth endoplasmic reticulum particularly in peripheral portions of cells, extensive formation of myelin figures and accumulation of lipid globules.

Groups 1, 4, 6 and 7. Certain additional changes were observed in remaining groups. In group 1 the necrosis was very extensive throughout the liver. By electron microscopy, the necrotic areas showed advanced disruption of cells with mitochondrial swelling and fragmentation of endoplasmic reticulum. In group 4, no electron microscopic changes were observed.

By one hour, in rats given 2 mg per kg of crude aflatoxin (group 6), nuclear alterations indistinguishable from those present at 24 hours in group 3 (0.45 mg per kg) were already apparent, but only a moderate proliferation of smooth endoplasmic reticulum was seen.

By 12 hours rats given 1 mg per kg of crude aflatoxin IP (group 7) showed increased proliferation of smooth endoplasmic reticulum and prominent dissociations of ribosomes from ergastoplasmic membranes (Fig. 11). In occasional cells, partially sequestered groups of mitochondria showed bleb-like evaginations imparting a "dumbbell" shape to their profile. The evaginations lacked cristae and dense matrix granules and were limited by a single membrane (Fig. 12). The nuclear alterations were similar to those in group 3 at 24 hours or in group 6 at 1 hour.

Monkeys. Light Microscopy. Liver of the monkey given 2.6 mg per kg of pure aflatoxin and sacrificed at 48 hours showed sinusoidal congestion with cell vacuolization. While individual cell necrosis and eosinophilic bodies were prominent (Figs. 13 and 14), occasionally extensive areas involving contiguous lobules were necrotic. A moderate neutrophil reaction accompanied the necrosis. Slight oval cell proliferation was present as was a mononuclear infiltration about the portal tracts. The periportal necrosis seen in rats was not observed.

The liver in the monkey given 0.45 mg per kg pure aflatoxin orally and sacrificed at 48 hours showed very occasional acidophilic cell necrosis and slight periportal loss of glycogen with moderate to marked accumulation of intracytoplasmic fat droplets throughout the lobule. The liver in the animal given 2.5 mg per kg pure aflatoxin and sacrificed at 48 hours showed extensive hemorrhagic necrosis with few cells remaining intact. Neutrophils were present throughout and were particularly numerous in portal tracts. There was marked loss of glycogen. The reticulin pattern was not altered conspicuously. In the monkey given 3 mg per kg pure aflatoxin orally, by 96 hours there was massive hemorrhagic necrosis with a prominent neutrophil reaction throughout the lobules and in portal tracts. In addition, early bile duct proliferation was apparent.

Electron Microscopy. In nuclei, changes comparable to those in the

rat were observed. The nucleolus was separated into zones with distinct fibrillar and granular components (Fig. 15) with the granules being somewhat larger, more discrete and dispersed than in the rat. In many nuclei, angular condensations measuring 0.5 to 1 μ were common; these were of uncertain origin and composition (Fig. 16).

The ultrastructural alterations in the cytoplasm of viable liver cells in the monkey, while essentially similar to those in rats, appeared less pronounced and varied. Lipid accumulation and vesicles of smooth endoplasmic reticulum were prominent. Significant mitochondrial abnormalities were not detected.

Biochemical Studies

The results of the biochemical studies (group 3) are shown in Tables III and IV. While the protein/DNA value decreased equally in both the homogenate and in the nuclear fraction, the fall of RNA/DNA ratios was predominantly in the cytoplasm. The nuclear RNA/DNA ratio decreased by only 8 per cent. Glycogen levels dropped markedly but no

TABLE III

BIOCHEMICAL CHANGES IN RAT LIVER *

	Control	24 Hours	48 Hours	72 Hours
	Total homogenate †			
DNA mg/gm wet liver	2.58 ±0.05	2.70 ±0.07	2.84 ±0.07	2.98 ±0.10
RNA mg/mg DNA	3.46 ±0.03	2.68 ±0.09	2.17 ±0.10	2.07 ±0.08
Protein mg/mg DNA	74 ±0.84	65 ±1.69	59 ±1.68	57 ±4.03
Glycogen mg/mg DNA	9.4 ±1.43	3.7 ±0.94	1.7 ±0.25	1.4 ±0.40
Lipids mg/mg wet liver	48 ±2.69	48 ±1.74	47 ±4.86	55 ±3.97
G-6-p-ase mg/mg DNA	4.9 ±0.22	3.8 ±0.08	2.9 ±0.29	2.9 ±0.48
	Isolated nuclear fraction †			
DNA mg/gm wet liver	1.47 ±0.08	1.38 ±0.01	1.34 ±0.12	1.43 ±0.07
RNA mg/mg DNA	0.33 ±0.01	0.29 ±0.01	0.28 ±0.01	0.30 ±0.003
Protein mg/mg DNA	5.1 ±0.24	4.8 ±0.48	4.5 ±0.19	3.8 ±0.28
G-6-p-ase mg/mg DNA	0.028 ±0.01	0.04 ±0.002	0.03 ±0.006	0.017 ±0.003

* Oral dose: 0.450 mg pure aflatoxin per kg body weight.
† Mean ± standard error.

TABLE IV

CHANGES IN RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA FOLLOWING AFLATOXIN *

	Number of animals	P:O ratios	$\mu l\ O_2$/mg N/hour
Controls	4	3.5±0.06	38±5
24 hours	4	2.9±0.12	28±6
48 hours	4	1.7±0.03	16±3
72 hours	4	1.4±0.04	34±5

* Oral dose: 0.450 mg pure aflatoxin per kg body weight.

uniform change was seen in lipid content. The low levels of glucose-6-phosphatase activity in the nuclear fractions indicated a high degree of purity of this fraction. In further studies, it was found that the degree of nuclear recovery (as determined by comparison of homogenate DNA to nuclear fraction DNA) correlated with the severity of necrosis as seen by light microscopy.

The results of studies on the mitochondrial fraction are shown in Table IV. Increasing functional damage to mitochondria with time is indicated by the decreasing P:O ratios after the administration of aflatoxin. Both oxygen consumption and phosphorylation were inhibited although the essentially normal oxygen consumption at 72 hours may have indicated recovery of respiration without concomitant return to normal of phosphorylation. Since the P:O ratio at 72 hours was not greatly altered from the 48-hour value and the oxygen consumption doubled, the absolute amount of phosphorylation per mg of N essentially doubled between 48 and 72 hours.

Chronic Experiments

Light Microscopy. In chronic experiments, the lesions were almost totally confined to parenchymal cells. Bile duct and oval cell proliferation was scant or absent even after 33 weeks. Cirrhosis or fibrosis was never observed even when tumors were present. By 10 weeks, a few scattered hyperbasophilic foci and occasional megalocytes began to appear (Fig. 17). The lesions did not increase in number markedly; the parenchymal pattern tended to become indistinct with variation of cell size and width of cords. Hyperplastic cells were intermingled with foci of atrophic basophilic cells. In some areas, lines of stress appeared suggesting irregular parenchymal hyperplasia. The first tumor appeared at 26 weeks in a liver showing relatively little architectural abnormality. After restoration to a normal diet at 33 weeks changes similar to those above persisted though hyperbasophilic areas were more prominent and nucleolar enlargement was conspicuous in many areas. By 52 weeks,

hepatomas had developed in 12 of 15 animals. These tumors were well differentiated hepatomas or adeno-hepatomas composed of large, clear cells with a compact pattern with circular or sinuous lumens as previously described.[4,18] Some appeared to be associated with hyperbasophilic foci although the tumor cells more nearly resembled clear cells often adjacent to hyperbasophilic collections. Tumors were not encapsulated but were distinctly demarcated from the surrounding liver by absence of cytoplasmic basophilia (Fig. 18), lines of stress and compressed reticulum (Fig. 19). With PAS stains, though many tumor cells had abundant amounts of glycogen, others did not (Fig. 20).

Electron Microscopy. The electron microscopic changes in chronic experiments were slight. In no animals were the nucleolar alterations encountered in the acute experiments observed. By 10 weeks, the only conspicuous abnormality in liver cells was the presence throughout the cytoplasm of irregular spaces, even in paranuclear locations; these appeared to arise from dilated tortuosities of bile canaliculi (Fig. 21). After 10 weeks, the only consistent change noted was an increased prominence of the smooth endoplasmic reticulum and occasional areas of focal cytoplasmic necrosis. Dissociation of ribosomes from ergastoplasmic membranes was not appreciable at any stage. Hyperplasia of smooth ER was the only change uniformly present in liver cells after restoration to the normal diet and in non-tumor areas of the tumor-bearing liver.

Tumors. Tumor cells were characterized by prominent collections of vesicles of smooth endoplasmic reticulum, fat granules and a paucity of dense granules in the mitochondrial matrix (Fig. 22). Occasional cells contained mitochondria with linear strata of crystalloids (Fig. 23). The "clear" cell pattern of tumors observed by light microscopy appeared due to the extensive dilatation of cisterns of endoplasmic reticulum (Fig. 24). The lumen spaces seen by light microscopy were vascular sinusoids, often without a continuous lining (Fig. 25).

Two features predominated in the nuclei of tumor cells. In many, there were focal interruptions of the external lamina of the nuclear envelope. Second, most nuclei, regardless of plane of section, lacked a distinct normal nucleolus. Instead, the nucleus contained large interchromatinic areas with dispersed fibrils, presumably DNA, and collections of granules compatible with interchromatin granules (Fig. 26).

Discussion

Acute Effects

The acute changes in the rat as viewed by conventional microscopy were essentially as described by others and included periportal parenchymal cell necrosis and slight bile duct proliferation.[5,19,20] With electron

microscopy, the most prominent features were separation of the granular and fibrillar components of the nucleolus with the formation of nucleolar "caps"[21,24] in both rats and monkeys. The morphologic alterations were accompanied by a slight decrease in nuclear RNA content, and a marked fall in cytoplasmic RNA. A significant fall in both nuclear and cytoplasmic protein was also observed.

The nomenclature of nucleolar constituents as observed by electron microscopy is based largely on the terminology developed from earlier light microscopic studies; consequently certain inconsistencies have resulted. The nucleoli in the present experiments showed such a wide variety of structural abnormalities that it was difficult to construct a temporal sequence of changes even at 1 hour. Further, it is uncertain whether these variations represented a continuum proceeding from a single biochemical cause or whether some changes were morphologic expressions of secondary or tertiary lesions. Current knowledge about nuclei does not permit definite conclusions at this time and, as Schoefl stated[22] if any nucleolar synthetic processes are blocked, the structure undergoes marked alteration.

It is generally agreed that the primary function of the nucleolus is related to ribosomal and protein synthesis. Chipchase and Birnstiel[25] concluded that most, if not all, of the ribosomal RNA is synthesized by non-nucleolar portions of chromatin and then transferred to the nucleolus for assembly into ribosomes. Autoradiographic studies[26] have also shown that nuclear RNA synthesis occurs in both the nucleolus and the chromatin with the former consistently containing more label than the latter. Accordingly, it is possible that the nucleolar changes may be a nonspecific morphologic representation of interference with the chromatin–nucleolar axis and that they reflect variations in fundamental cell activity, particularly protein synthesis.

The broader implications of the nuclear alterations due to aflatoxin are difficult to envisage. Similar morphologic changes are caused by a variety of agents whose biochemical action on cell nuclei either differs from that of aflatoxin or has not yet been studied adequately to permit functional generalization. Similar nuclear changes have been reported after actinomycin D, mitomycin, 4-nitroquinoline-N-oxide and certain pyrrolizidine alkaloids.[22–24,27] Despite differences in time of onset, duration and pattern, they appear to be essentially of the same order in all cases, and one may speculate further on their functional significance.

The pyrrolizidine alkaloids, lasiocarpine and Crotolaria extract, cause an early decrease in total cell content of RNA and protein and probably act as alkylating agents.[28] They also induce nucleolar abnormalities indistinguishable from those due to aflatoxin. It is therefore possible that

nucleolar "caps" represent morphologic evidence of alkylation.[27] However, not all alkylating agents induce similar nucleolar alterations. Other agents which methylate, such as dimethylnitrosamine,[29] or ethylate RNA and possibly DNA[30,31] do not appear to cause such abnormalities.[32,33] Also, comparisons of actinomycin D, mitomycin C, and 4-nitroquinoline-N-oxide reveal certain differences in their effects on cells and inconsistencies that do not permit assigning a single cause for nucleolar "caps". Actinomycin causes rapid, early and extensive inhibition of DNA-dependent RNA synthesis. On the other hand, with mitomycin, RNA synthesis is not disturbed initially although later there is a moderate action on RNA and protein synthesis.[34,35] Nitro-quinoline-N-oxide, moreover, primarily induces SH-containing enzymes followed by arrest of glycolysis and decreased cellular ATP content with inhibition of nucleic acid and protein synthesis following secondarily.[23] It has been suggested that mitomycin cause a cross-linking of DNA strands or depolymerization of DNA. Preliminary studies in this laboratory, however, using large doses of nalidixic acid, a drug which resembles radiomimetic agents and which cross-links DNA strands yielding a DNA resembling that isolated from cells treated with alkylating agents,[36] failed to produce nucleolar changes within 24 hours.[37] Furthermore, x-radiation of cells produces nuclear and nucleolar alterations that bear little resemblance to those due to aflatoxin, lasiocarpine or mitomycin.[38]

Clifford and Rees[39] showed that 30 minutes after aflatoxin poisoning in rats, one-third of the cell content of aflatoxin was in the nucleus. It was further shown that calf thymus DNA altered the absorption spectrum of aflatoxin B_1. Similarly, Sporn, Dingman, Phelps and Wogan[40] showed that aflatoxin binds to DNA and lowers nuclear RNA content. The latter workers pointed out that the characteristics of enzyme inhibition caused by aflatoxin were very similar to those caused by actinomycin D.

The nucleolar zonal rearrangement could have been a result of increased nuclear RNase activity. Inhibitors of RNase in cell nucleoli have been demonstrated but their role is largely unknown.[41] Chevremont, Chevremont-Comhaire and Firket[42] reported, by light microscopy of cultured cells, that RNase caused shrinkage and fragmentation of nucleoli; no electron microscopic studies have been reported, however, in intact animals. A further possibility is interference with the normal function of histones which appear to play an important role in DNA repression,[43] (Allfrey, cited by Moore[44]), and in the regulation of nucleolar RNA synthesis.[45] Conceivably, interruption of the normal stabilizing effect of histones[46] could result in abnormal nuclear structure.

It would appear that zonal transformation itself is a secondary change probably depending on altered nuclear constituents other than the nucleolus since it occurs after ultraviolet radiation of non-nucleolar areas of the nucleus rich in DNA, but not after radiation of the nucleolus itself.[47] It has been suggested that nucleolar "caps" in cell cultures represent a form of spontaneous degeneration.[48] Long-term protein deficiency causes appreciable zonal change in nucleolar constituents.[49]

In conclusion, it would appear that zonal transformation is a secondary change in the nucleolus dependent upon a disturbance in the configuration of DNA, histones or RNase and results in a consequent alteration in RNA and protein synthesis.

Biochemical Effects

Although only limited studies of the biochemical effects of aflatoxin are available, certain interesting aberrations in liver cell activity have been reported. Wogan and Friedman[50] showed that aflatoxin interfered with the ability of liver cells to respond to enzyme induction after the administration of tryptophan or hydrocortisone. Friedman and Wogan[51] and Sporn and co-workers[40] also showed that aflatoxin B_1 caused a marked reduction in the nuclear RNA/DNA ratio. These workers indicated[51] that the early effect on RNA metabolism could be one of the primary manifestations of aflatoxin toxicity. On the other hand, Frayssinet, Lafarge, DeRecondo and LeBreton[52] found no impairment of RNA synthesis in the nucleus following aflatoxin. In our experiments, while the RNA/DNA ratio in the homogenate fell by 40 per cent, that in the nuclear fraction decreased by only 8 per cent. It is possible that the higher dose of aflatoxin used by Wogan and colleagues caused more extensive cell necrosis with nuclear disruption permitting leakage of RNA out of the nuclei. Additional experiments in our laboratories with aflatoxin have demonstrated that the percent recovery in preparing the nuclear fraction by centrifugation, as measured by DNA content, is directly related to the extent of necrosis. Recovery thus probably relates to the proportion of intact nuclei within a homogenate.

Wogan[53] also showed that there was a biphasic response in protein synthesis following aflatoxin; an immediate, transient suppression was followed, at 3 days, by prolonged stimulation for several days. Our studies, dealing only with protein content rather than synthesis or turnover, showed that the protein/DNA ratio in the homogenate fell by 24 per cent. Frayssinet and co-workers[52] showed that aflatoxin given to rats 5 days before partial hepatectomy inhibited liver regeneration and caused a decrease in total RNA and protein synthesis.

Chronic Effects

In view of the potent carcinogenic properties of aflatoxin which may cause tumors even following a single dose, the possible relationship of the acute toxic changes to the chronic carcinogenic properties of the agent are of interest. The acute morphologic changes occurring in the nucleus were not observed in the chronic studies and nuclear and cytoplasmic alterations were negligible. This is in conformity with the light microscopic findings in which liver damage was minimal prior to the onset of small irregular adenomatous nodules. Although the present studies support the role of a direct nuclear effect by aflatoxin, significant nucleolar abnormalities in chronic poisoning, prior to appearance of tumors, were not observed. The relationship of acute nucleolar changes to carcinogenesis becomes problematic when one considers that potent carcinogens, such as polycylic hydrocarbons and azo dyes, act predominantly in the cytoplasm; other agents which interact with DNA or destroy the chemical integrity of chromosomes may be only weakly active or inert as carcinogens. If the acute nuclear changes are related to the carcinogenic process, it is possible that they occur in only a few cells which were missed on electron microscopic sampling of the liver during chronic experiments.

Other Species

In the monkey, Madhavan, Suryanarayana and Tulpule[54] showed that aflatoxin caused severe, generalized fatty change, proliferation of ductular cells, periductular sheaths of connective tissue and intralobular and portal clusters of mononuclear cells with little necrosis or regeneration of hepatic cells.

In the present study, the light microscopic changes in monkey liver in acute poisoning were distinctly different from those seen in the rat. Focal cell necrosis predominated in contrast to the marked periportal necrosis present in the rat and the duckling.[55] The pattern of these changes was not unlike the lesions of viral hepatitis in man, except for the lack of periportal cellular infiltration. This aspect, however, requires further study at various dose levels. The observations in simian primates raise the possibility that, in man, an episode of aflatoxin poisoning could be confused, on histologic grounds, with acute viral hepatitis. The possibility becomes all the more interesting when one considers that viral infection of the liver has been linked to some liver cancers in Africa,[2,10] and that periportal hepatic necrosis such as occurs in the rat is rarely seen in populations among whom there is a high incidence of liver cancer.

In the duckling, Theron [55] demonstrated lysis of mitochondrial membranes 4 hours after administration of aflatoxin. He illustrated, in addition to mitochondrial abnormalities, a decrease in membrane-associated enzymes as well as structural damage in membranes of mitochondria, microvilli, or endoplasmic reticulum in close contact with extravasated erythrocytes. He stated that the toxin was transported by erythrocytes and, accordingly, all membrane structures in close contact with toxin-bearing red cells were injured. Such intracellular effects of red cells were not seen by us in either rat or monkey liver.

Summary

Acute studies of ultrastructural and biochemical abnormalities induced by aflatoxin B_1 in the liver of rats and monkeys revealed abnormalities in nuclear fine structure. These were accompanied by decreased cytoplasmic RNA and protein content as well as a fall in nuclear protein levels. The early abnormalities in nuclear ultrastructure resembled those due to actinomycin and other agents which cause nuclear "capping". The biochemical lesion primarily related to the nucleolar responses, however, requires further elucidation. Mitochondrial function, as determined by P:O ratios, was abnormal in early stages following aflatoxin administration, but it appeared that respiratory activity recovered without a simultaneous and comparable degree of phosphorylative capacity.

The acute hepatic lesions in monkeys, by light microscopy, bore some resemblance to the changes in human liver in acute viral hepatitis.

In chronic experiments, nucleolar changes such as those present in acute experiments were not observed. On the other hand, well differentiated hepatocellular carcinomas appeared in a significant number of animals whose livers otherwise showed little architectural abnormality.

References

1. Dickens, F., and Jones, H. E. The carcinogenic action of aflatoxin after its subcutaneous injection in the rat. *Brit. J. Cancer,* 1963, 17, 691–698.
2. Kraybill, H. F., and Shimkin, M. B. Carcinogenesis Related to Foods Contaminated by Processing and Fungal Metabolites. In: Advances in Cancer Research. Haddow, A., and Weinhouse, S. (eds.). Academic Press, New York, 1964, Vol. 8, pp. 191–248.
3. Allcroft, R., and Carnaghan, R.B.A. Toxic products in groundnuts. Biological effects. *Chem. Industr. (London),* 1963, 50–53.
4. Butler, W. H., and Barnes, J. M. Toxic effects of groundnut meal containing aflatoxin to rats and guinea-pigs. *Brit. J. Cancer,* 1963, 17, 699–710.
5. Butler, W. H. Liver Injury and Aflatoxin; In: Mycotoxins in Foodstuffs. Wogan, G. N. (ed.). The M.I.T. Press, Cambridge, Mass., 1965, pp. 175–186.
6. Schoental, R. Liver changes and primary liver tumours in rats given toxic guinea pig diet (M.R.C. diet 18). *Brit. J. Cancer,* 1961, 15, 812–815.

7. De Iongh, H.; Beerthuis, R. K.; Vles, R. O.; Barrett, C. B., and Ord, W. O. Investigation of the factor in groundnut meal responsible for "turkey X disease." *Biochim. Biophys. Acta.*, 1962, **65**, 548–551.

8. Nesbitt, B. F.; O'Kelly, J.; Sargeant, K., and Sheridan, A. *Aspergillus flavus* and turkey X disease. Toxic metabolites of *Aspergillus flavus*. *Nature (London)*, 1962, **195**, 1062–1063.

9. Smith, R., and McKernan, W. Hepatotoxic action of chromatographically separated fractions of *Aspergillus flavus* extracts. *Nature (London)*, 1962, **195**, 1301–1303.

10. Oettlé, A. G. Cancer in Africa, especially in regions south of the Sahara. *J. Nat. Cancer Inst.*, 1964, **33**, 383–439.

11. Widnell, C. C., and Tata, J. R. A procedure for the isolation of enzymically active rat-liver nuclei. *Biochem. J.*, 1964, **92**, 313–317.

12. Swanson, M. A. Glucose-6-phosphatase from Liver. In: Methods in Enzymology. Colowick, S. P., and Kaplan, N. O. (eds.). Academic Press, New York, 1955, Vol. II, pp. 541–543.

13. Hawk, P. B.; Oser, B., and Summerson, W. Practical Physiological Chemistry. Blakiston, New York, 1954, ed.13, pp. 630–633.

14. Schneider, W. C. Determination of Nucleic acids in Tissues by Pentose Analysis. In: Methods in Enzymology. Colowick, S. P., and Kaplan, N. O. (eds.). Academic Press New York, 1957, Vol. III, pp. 680–684.

15. Layne, E. Spectrophotometric and Turbidimetric Methods for Measuring Proteins. In: Methods in Enzymology. Colowick, S. P., and Kaplan, N. O. (eds.). Academic Press, New York, 1957, Vol. III, p. 448.

16. Seifter, S.; Dayton, S.; Novic, B., and Muntwyler, E. The estimation of glycogen with the anthrone reagent. *Arch. Biochem.*, 1950, **25**, 191–200.

17. Umbreit, W. W.; Burris, R. H., and Stauffer, J. F. The Homogenate Technique. In: Manometric Technique. Burgess Publishing Co., Minneapolis, Minn., 1964, ed. 4, pp. 159–176.

18. Newberne, P. M. Carcinogenicity of Aflatoxin-contaminated Peanut Meals. In: Mycotoxins in Foodstuffs. Wogan, G. N. (ed.). M.I.T. Press, Cambridge, Mass., 1965, pp. 187–208.

19. Madhavan, T. V., and Gopalan, C. Effect of dietary protein on aflatoxin liver injury in weanling rats. *Arch. Path. (Chicago)* 1965, **80**, 123–126.

20. Tulpule, P. G.; Madhavan, T. V., and Gopalan, C. Effect of feeding aflatoxin to young monkeys. *Lancet*, 1964, **1**, 962–963.

21. Bernhard, W.; Frayssinet, C.; Lafarge, C., and Le Breton, É. Lésions nucléolaires précoces provoquées par l'aflatoxine dans les cellules hépatiques du rat. *C. R. Acad. Sci. (Paris)*, 1965, **261**, 1785–1788.

22. Schoefl, G. I. The effect of actinomycin D on the fine structure of the nucleolus. *J. Ultrastruct. Res.*, 1964, **10**, 224–243.

23. 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.

24. Reynolds, R. C.; Montgomery, P. O'B., and Karney, D. Nucleolar "caps"—a morphologic entity produced by the carcinogen 4-nitroquinoline-N-oxide. *Cancer Res.*, 1963, **23**, 535–538.

25. Chipchase, M. I., and Birnstiel, M. L. On the nature of nucleolar RNA. *Proc. Nat. Acad. Sci. (USA)*, 1963, **50**, 1101–1107.

26. Karasaki, S. Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J. Cell Biol.*, 1965, **26**, 937–958.

27. SVOBODA, D., and SOGA, J. Early effects of pyrrolizidine alkaloids on the fine structure of rat liver cells. *Amer. J. Path.*, 1966, 48, 347–373.

28. CULVENOR, C. C.; DANN, A. T., and DICK, A. Alkylation as the mechanism by which the hepatotoxic pyrrolizidine alkaloids act on cell nuclei. *Nature (London)*, 1962, 195, 570–573.

29. MAGEE, P. N., and FARBER, E. Toxic liver injury and carcinogenesis. Methylation of rat-liver nucleic acids by dimethylnitrosamine *in vivo*. *Biochem. J.*, 1962, 83, 114–124.

30. FARBER, E., and MAGEE, P. N. The probable alkylation of liver ribonucleic acid by the hepatic carcinogens dimethylnitrosamine and ethionine. *Biochem. J.*, 1960, 76, 58P.

31. STEKOL, J. A.; MODY, U., and PERRY, J. The incorporation of the carbon of the ethyl group of ethionine into liver nucleic acids and the effect of ethionine feeding on the content of nucleic acids in rat liver. *J. Biol. Chem.*, 1960, 235, PC59–PC60.

32. EMMELOT, P., and BENEDETTI, E. L. Changes in the fine structure of rat liver cells brought about by dimethylnitrosamine. *J. Biophys. & Biochem. Cytol.*, 1960, 7, 393–396.

33. LOMBARDI, B. Considerations on the pathogenesis of fatty liver. *Lab. Invest.*, 1966, 15, 1–15.

34. KURODA, Y., and FURUYAMA, J. Physiological and biochemical studies of effects of mitomycin C on strain HeLa cells in cell culture. *Cancer Res.*, 1963, 23, 682–687.

35. SCHWARTZ, H. S.; STERNBERG, S. S., and PHILIPS, F. Pharmacology of mitomycin C. IV. Effects *in vivo* on nucleic acid synthesis. Comparison with actinomycin D. *Cancer Res.*, 1963, 23, 1125–1136.

36. ROSENKRANZ, H. S., and LAMBETZ, C. *In vivo* effect of nalidixic acid (neg Gram) on the DNA of human diploid cells in tissue culture. *Proc. Soc. Exp. Biol. Med.*, 1965, 120, 549–552.

37. SVOBODA, D., and RACELA, A. Unpublished observations.

38. MONTGOMERY, P. O'B.; KARNEY, D.; REYNOLDS, R. C., and MCCLENDON, D. Cellular and subcellular effects of ionizing radiations. *Amer. J. Path.* 1965, 44, 727–746.

39. CLIFFORD, J., and REES, K. Aflatoxin: a site of action in the rat liver cell. *Nature (London)*, 1966, 209, 312–313.

40. SPORN, M. B.; DINGMAN, C. W.; PHELPS, H. L., and WOGAN, G. Aflatoxin B_1: binding to DNA *in vitro* and alteration of RNA metabolism *in vivo*. *Science*, 1966, 151, 1539–1541.

41. BUSCH, H.; BYVOET, P., and SMETANA, K. The nucleolus of the cancer cell: a review. *Cancer Res.*, 1963, 23, 313–339.

42. CHEVREMONT, M.; CHEVREMONT-COMHAIRE, S., and FIRKET, H. Étude de l'action de la ribonuclease sur des cellules vivantes cultivées *in vitro* et en particulier de ses effets sur la mitose. *Arch.Biol.(Liege)*, 1956, 67, 635–656.

43. HNILICA, L. S.; LIAU, M. C., and HURLBERT, R. B. Biosynthesis and composition of histones in Novikoff hepatoma nuclei and nucleoli. *Science*, 1966, 152, 521–523.

44. MOORE, R. D. Gene activation. *Science*, 1966, 152, 548–549.

45. LIAU, M. C.; HNILICA, L. S., and HURLBERT, R. B. Regulation of RNA synthesis in isolated nucleoli by histones and nucleolar proteins. *Proc. Nat. Acad. Sci. (USA)*, 1965, 53, 626–632.

46. BUSCH, H. Histones and Other Nuclear Proteins. Academic Press, New York, 1965.

47. MONTGOMERY, P. O'B.; REYNOLDS, R. C., and MCCLENDON, D. E. Nucleolar "caps" produced by ultraviolet flying spot irradiation. *J. Cell Biol.*, 1965, 27, 69A.

48. JÉZÉQUEL, A-M., and STEINER, J. W. Nucleolar caps—a spontaneous form of cellular degeneration? (Abstract) *Fed. Proc.*, 1966, 25, 667.

49. SVOBODA, D., and HIGGINSON, J. Ultrastructural changes produced by protein and related deficiencies in the rat liver. *Amer. J. Path.*, 1964, 45, 353–379.

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

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

52. FRAYSSINET, C.; LAFARGE, C.; DE RECONDO, A-M., and LE BRETON, E. Inhibition of liver compensatory hypertrophy in the rat by *Aspergillus flavus* toxins. *C. R. Acad. Sci. (Paris)*, 1964, 259, 2143–2146.

53. WOGAN, G. N. Experimental Toxicity and Carcinogenicity of Aflatoxins. In: Mycotoxins in Foodstuffs. WOGAN, G. N. (ed.). The M.I.T. Press, Cambridge, Mass., 1965, pp. 163–173.

54. MADHAVAN, T. V.; SURYANARAYANA, R. K., and TULPULE, P. G. Effect of dietary protein level on susceptibility of monkeys to aflatoxin liver injury. *Indian J. Med. Res.*, 1965, 53, 984–989.

55. THERON, J. J. Acute liver injury in ducklings as a result of aflatoxin poisoning. *Lab. Invest.*, 1965, 14, 1586–1603.

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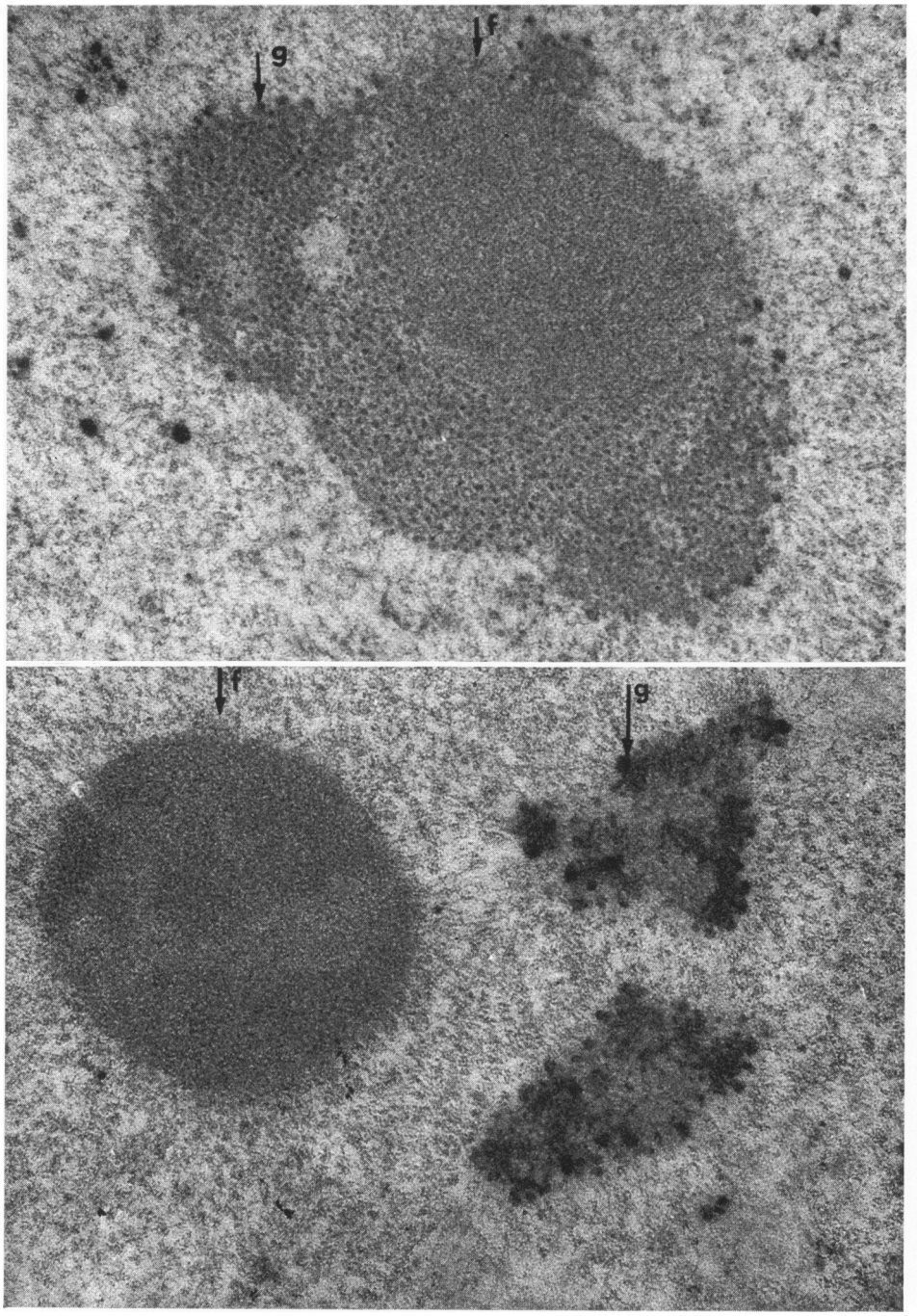
LEGENDS FOR FIGURES

Figures 1 through 10 are from the livers of rats given 0.45 mg per kg of pure aflatoxin orally.

FIG. 1. Twenty-four hours. The fibrillar (f) and granular (g) components of the nucleolus are separated into two distinct zones. Lead stain. × 60,000.

FIG. 2. Twenty-four hours. There is complete separation of the fibrillar (f) and granular (g) components with some dispersion of nucleolus-associated chromatin. Lead stain. × 60,000.



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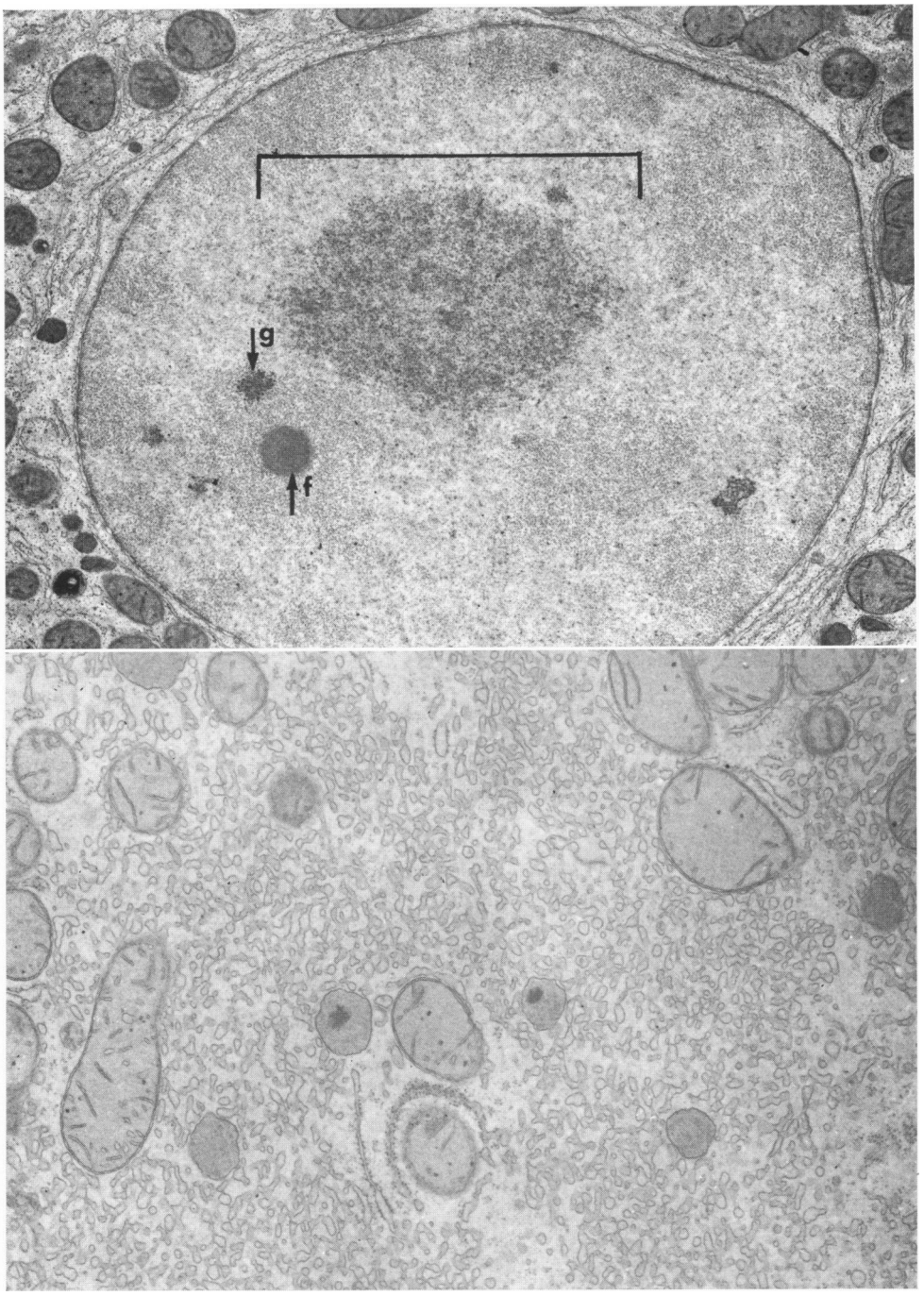

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FIG. 3. Twenty-four hours. Nucleolar constituents are separated (f,g) and accompanied by a constellation of granules (bracket). The granular constellation probably does not represent a "burst" nucleolus [22] since the fibrillar and granular nucleolar constituents are still present. Lead stain. × 9,900.

FIG. 4. Twenty-four hours. There is marked proliferation of vesicles of smooth endoplasmic reticulum. Lead stain. × 9,900.

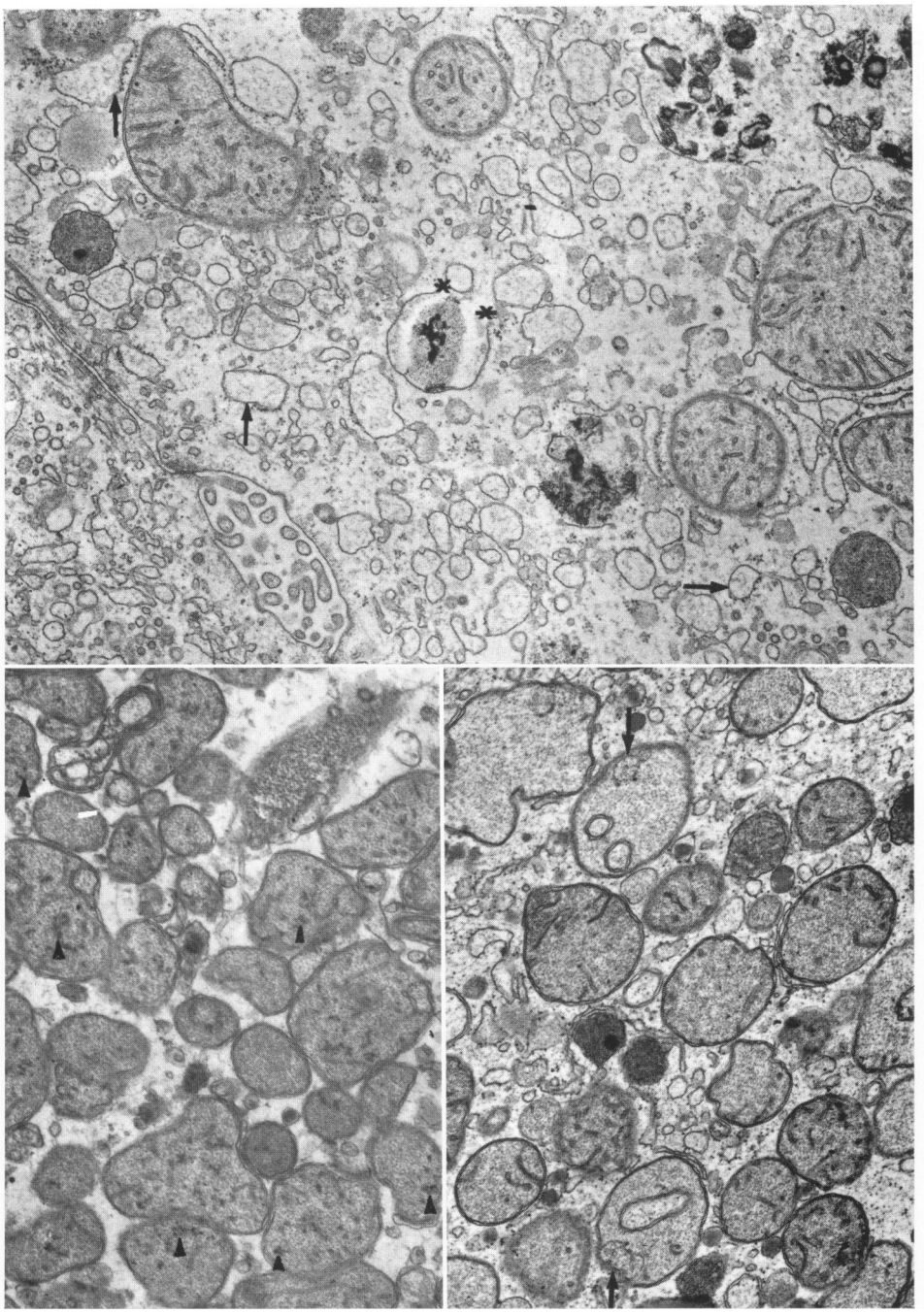

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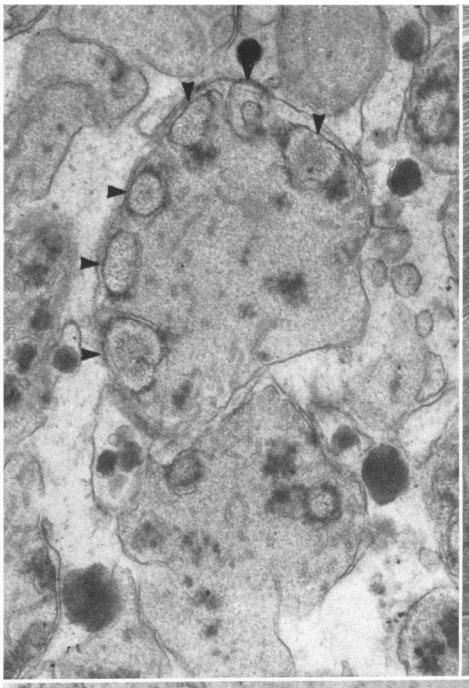
FIG. 5. Twenty-four hours. Dilatation and partial degranulation (arrows) of the endoplasmic reticulum are prominent. Early focal cytoplasmic necrosis is present; the foci are only partially limited by a membrane (asterisks). Lead stain. × 17,000.

FIGS. 6 and 7. Twenty-four hours. The mitochondria, though lacking normal dense matrix granules, contain ill-defined flecks (arrows). In Figure 7, selected mitochondria with membrane-limited matrix "cavities" are illustrated. Lead stain. × 17,000.

FIG. 8. Forty-eight hours. In the matrix of many mitochondria of periportal cells, there are several roughly circular areas within a granular interior and usually a more dense periphery (arrows). Lead stain. × 28,800.

FIG. 9. Extensive development of the smooth endoplasmic reticulum forms myelin figures. This appears in many cells at 48 hours. Continuity with the rough endoplasmic reticulum is apparent at the arrow. Lead stain. × 11,400.

FIG. 10. In the midzonal and centrilobular cells the mitochondria frequently exhibit indentations (i). Numerous lipid droplets (l) are present in cisterns of endoplasmic reticulum Lead stain. × 17,000.



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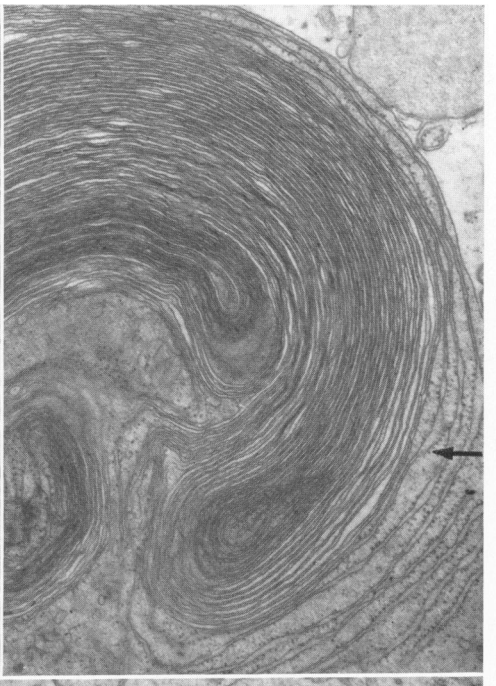

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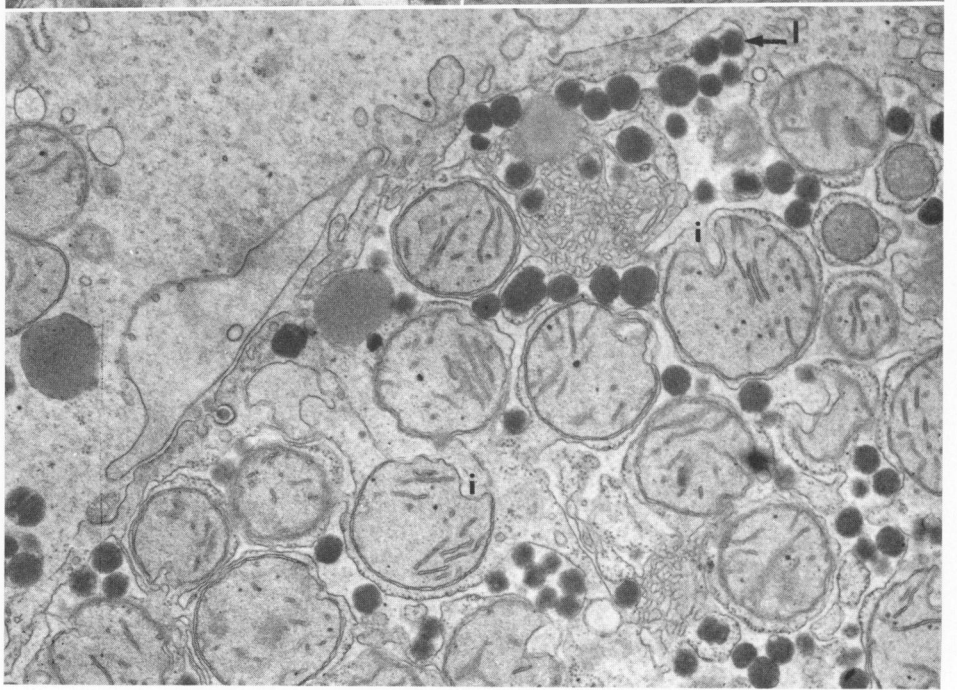

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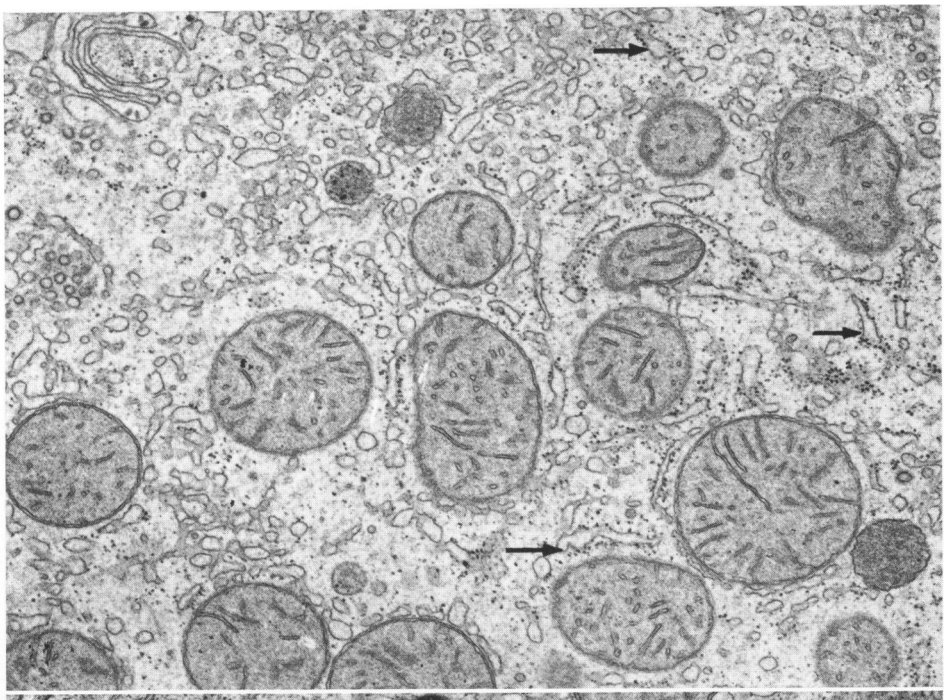

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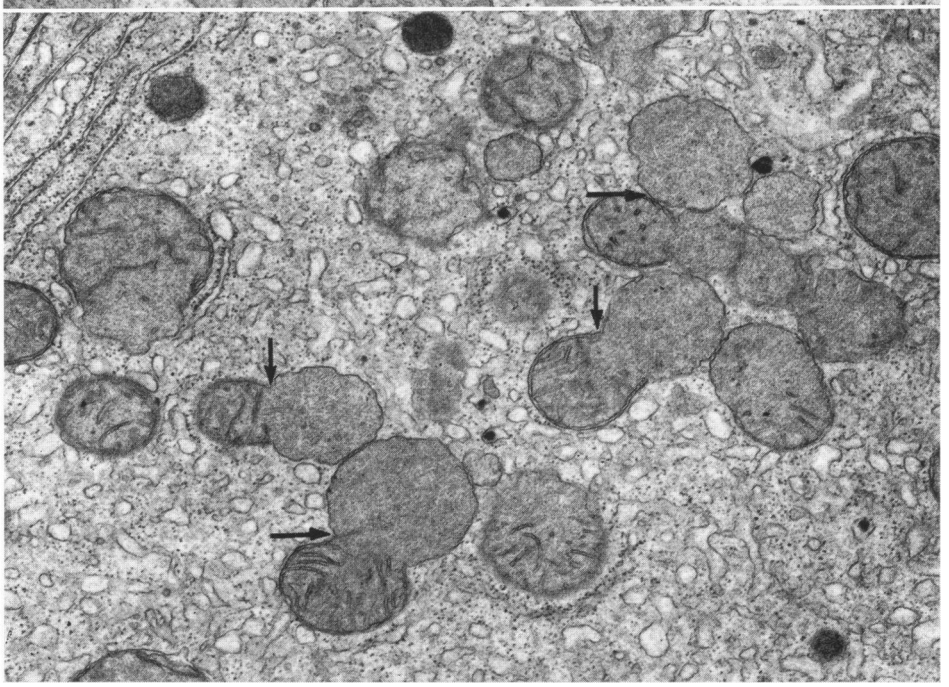

FIG. 11. In rats given 1 mg per kg crude aflatoxin, dissociation of ribosomes from ergastoplasmic membranes (arrows) is a conspicuous abnormality at 12 hours. Lead stain. × 20,000.

FIG. 12. At 12 hours after 1 mg per kg crude aflatoxin, mitochondria in partially sequestered portions of cytoplasm exhibit evaginated blebs (arrows) limited by a single membrane and lacking cristae and matrix granules. Lead stain. × 17,000.

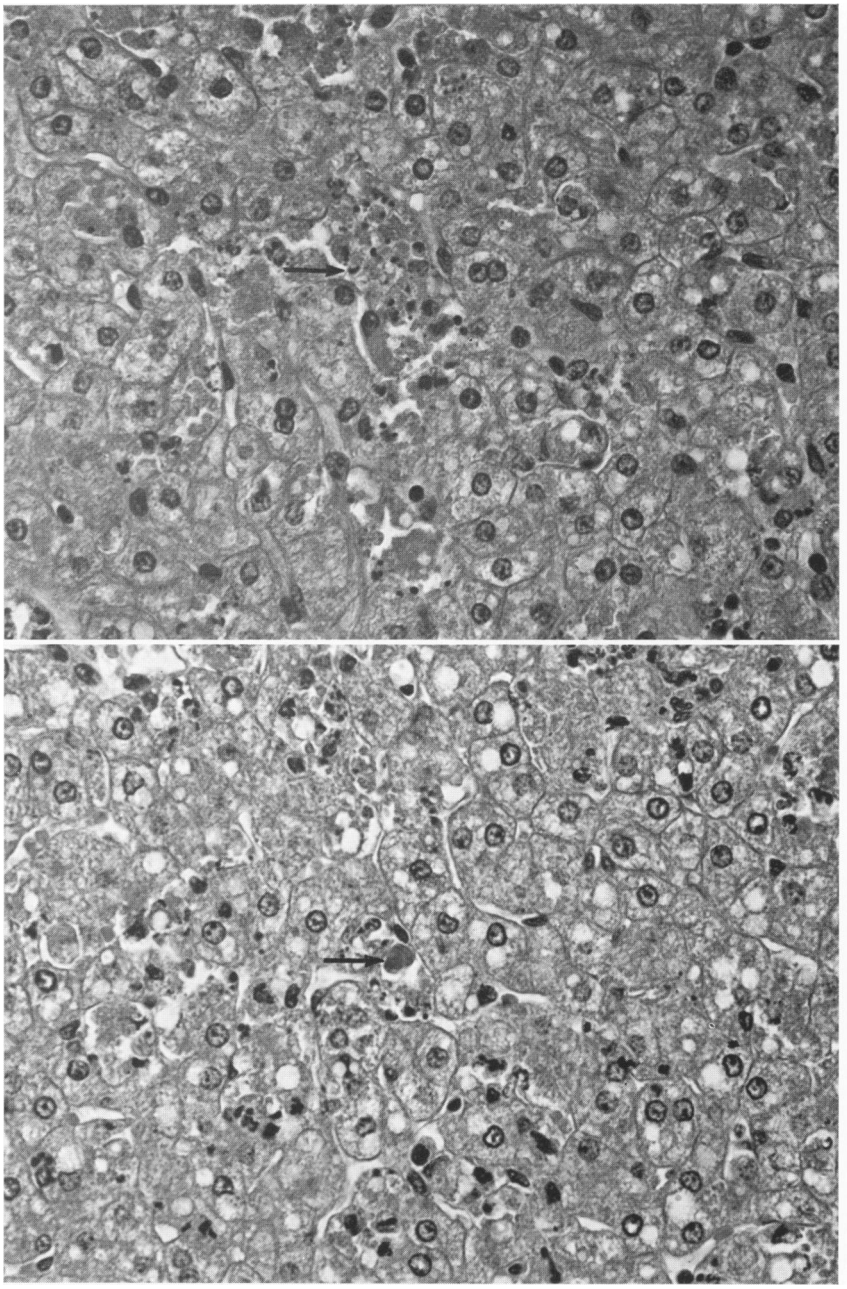

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FIG. 13. Monkey liver, 48 hours after 2.6 mg per kg pure aflatoxin. Necrosis of contiguous cells is evident along with nuclear debris (arrows). Hematoxylin and eosin stain. × 750.

FIG. 14. Monkey liver, 48 hours after 2.6 mg per kg pure aflatoxin. Isolated foci of cellular necrosis and homogeneous acidophilic bodies (arrow) are apparent. Hematoxylin and eosin stain. × 750.

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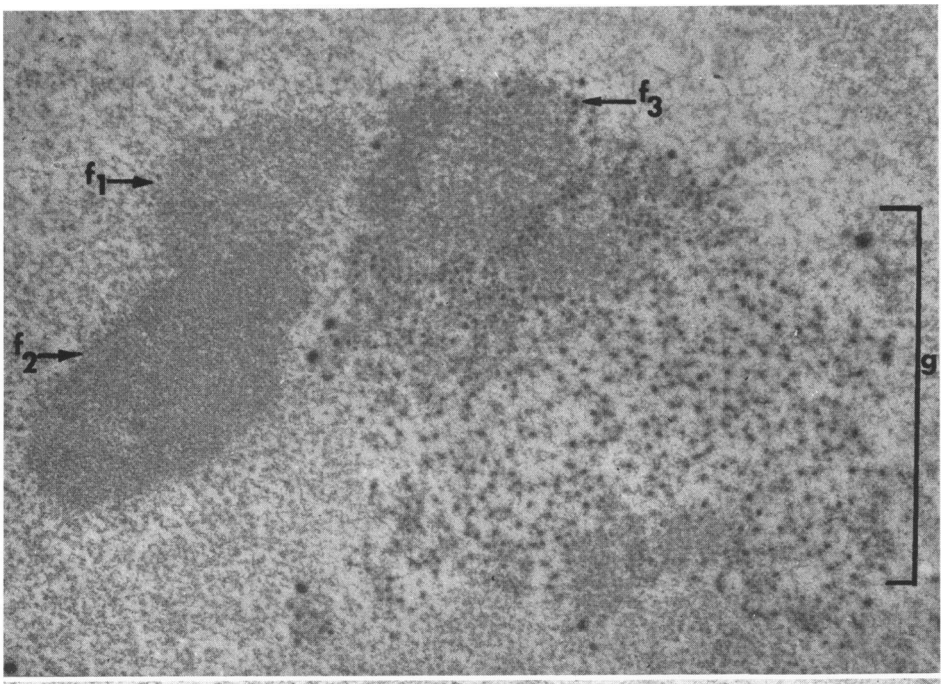

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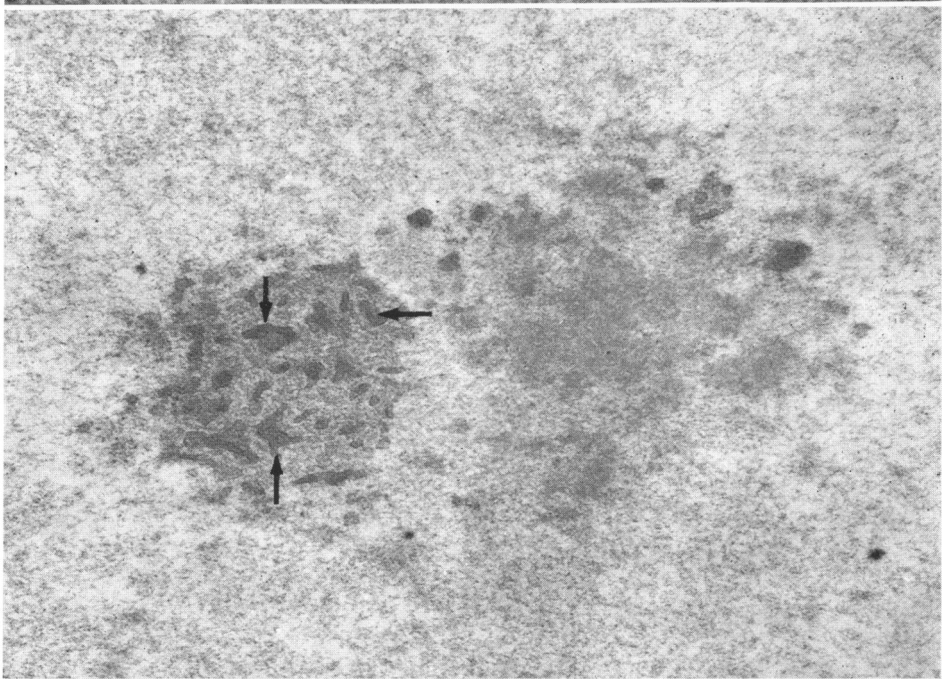
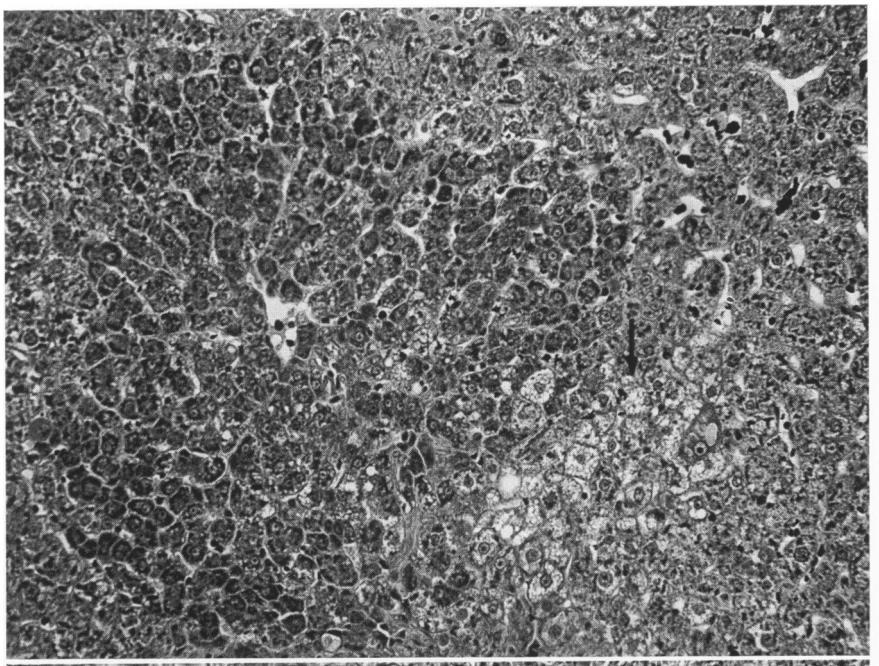


FIG. 15. Monkey liver 48 hours after 2.6 mg per kg pure aflatoxin. Zonal rearrangement of nucleolar constituents is prominent. The fibrillar components are present at f_1, f_2 and f_3 while granules (g) are large, discrete and separated. Lead stain. × 55,000.

FIG. 16. In many nucleoli of monkey liver cells 48 hours after aflatoxin, angular densities (arrows) are prominent. Lead stain. × 60,000.



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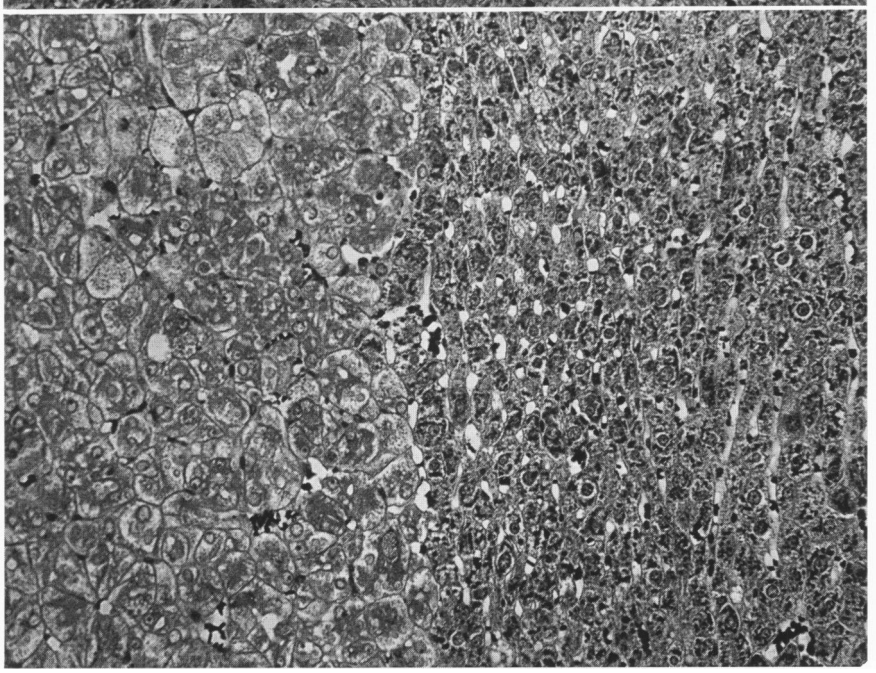

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FIG. 17. Tumors appear to be related to vacuolated cells (arrow) situated adjacent to hyperbasophilic foci. These are common in the rat liver after 16 weeks on a diet containing 1 ppm aflatoxin. Azure–eosin stain. × 250.

FIG. 18. Hepatoma, rat liver. Most tumors (left) are composed of large, clear cells in a compact pattern with occasional circular or elongated vascular channels. Tumor cells are distinctly demarcated from the non-tumor area (right) by the absence of cytoplasmic basosphilia. Azure–eosin stain. × 250.

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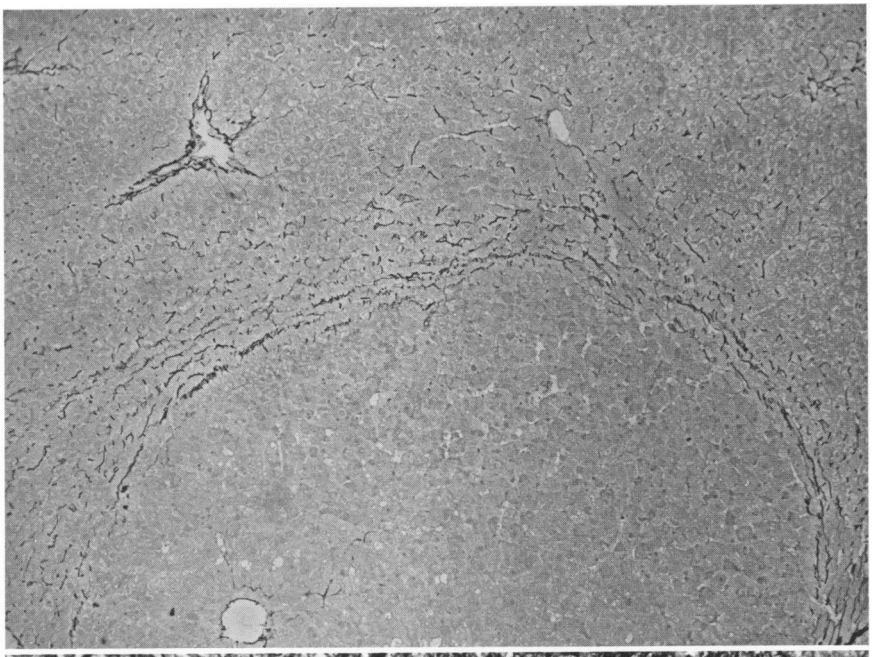

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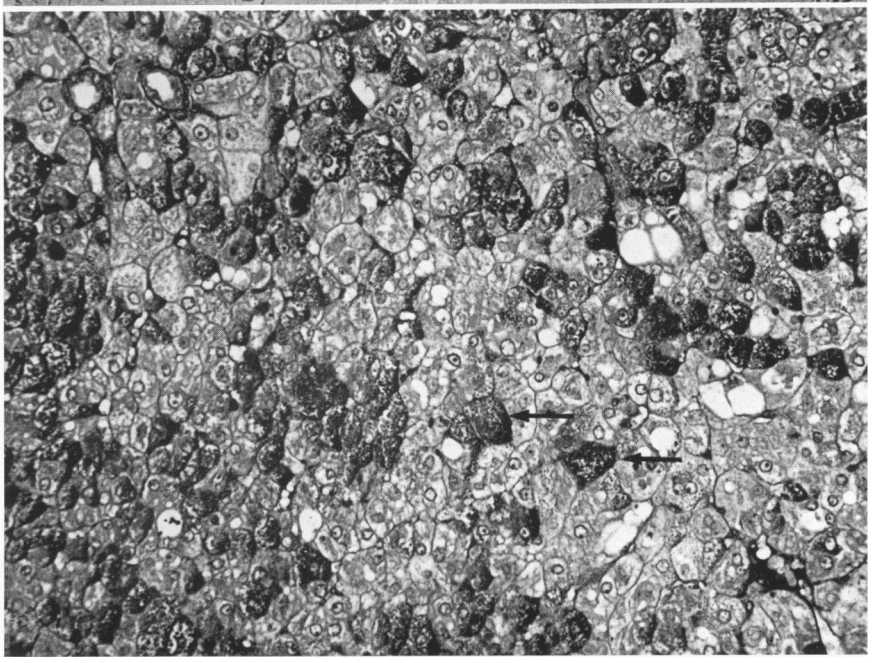
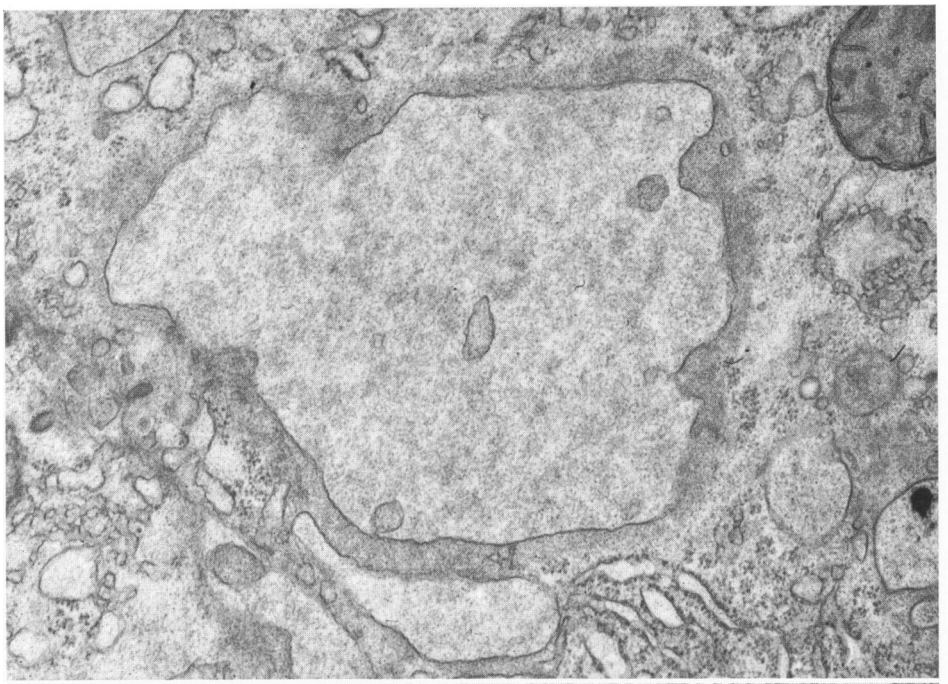


FIG. 19. Tumors are demarcated from the surrounding liver by a compressed reticulum at their periphery. Gordon–Sweet stain. × 100.

FIG. 20. Many tumor cells contain glycogen (arrows) while others do not. PAS stain with diastase digestion. × 250.



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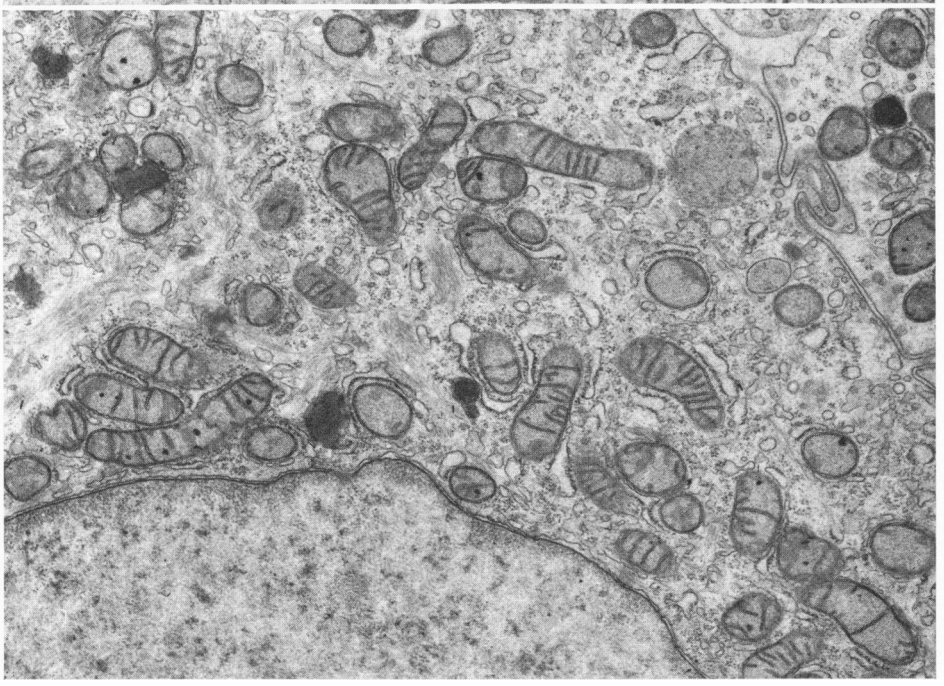

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FIG. 21. By 10 weeks, the most conspicuous abnormality in rat liver cells is the presence of intracytoplasmic spaces that appear to arise from dilated tortuosities of bile canaliculi. Lead stain. × 24,000.

FIG. 22. Tumor cells are characterized by poorly developed endoplasmic reticulum and a paucity or absence of dense granules in the mitochondrial matrix. Lead stain. × 14,200.

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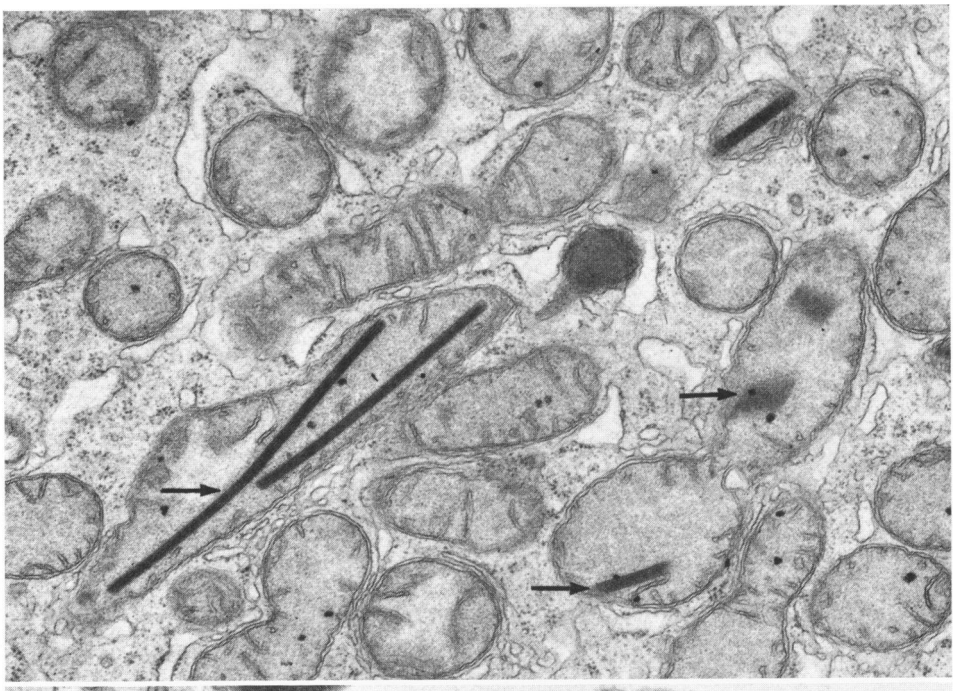

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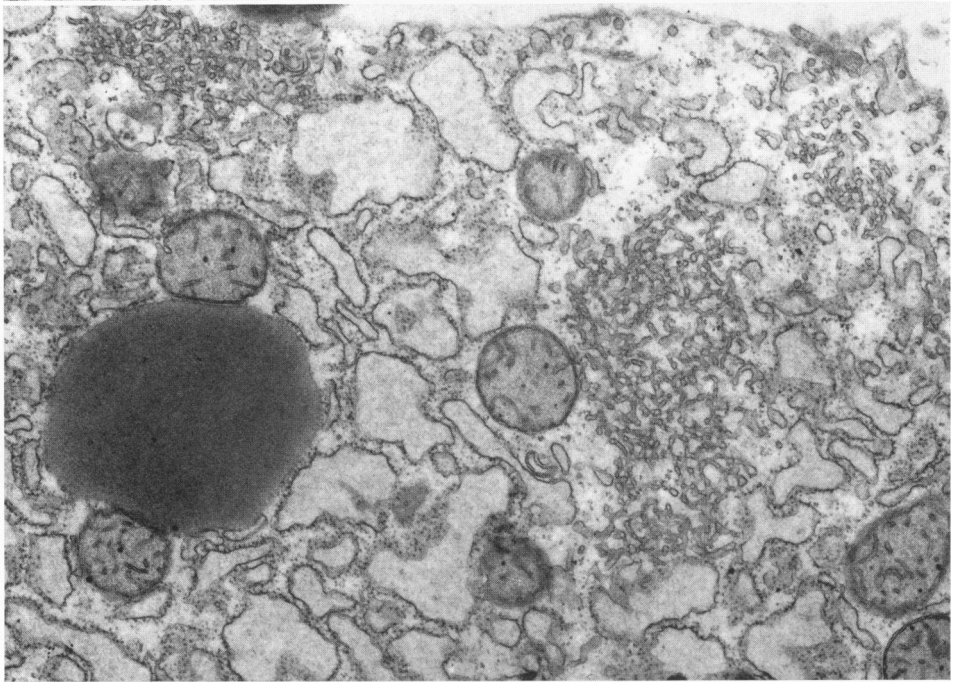
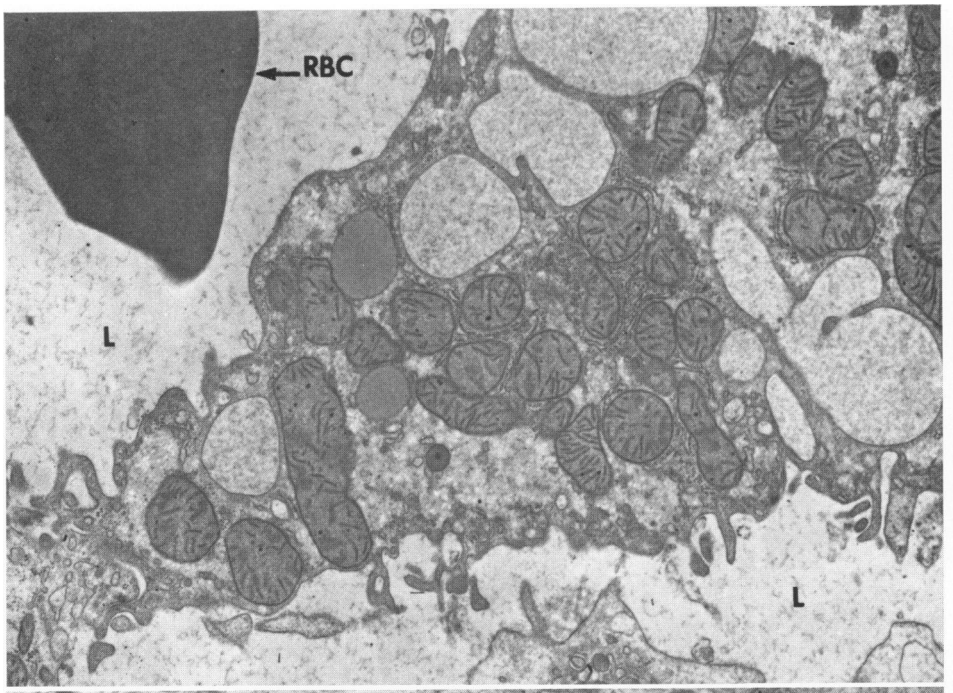


FIG. 23. The mitochondria in occasional tumor cells contain dense linear strata resembling crystalloids (arrows). Lead stain. × 24,000.

FIG. 24. Throughout the cytoplasm of tumor cells, the most consistent change is dilatation of the cisterns and collections of vesicles of smooth ER. Lead stain. × 17,000.



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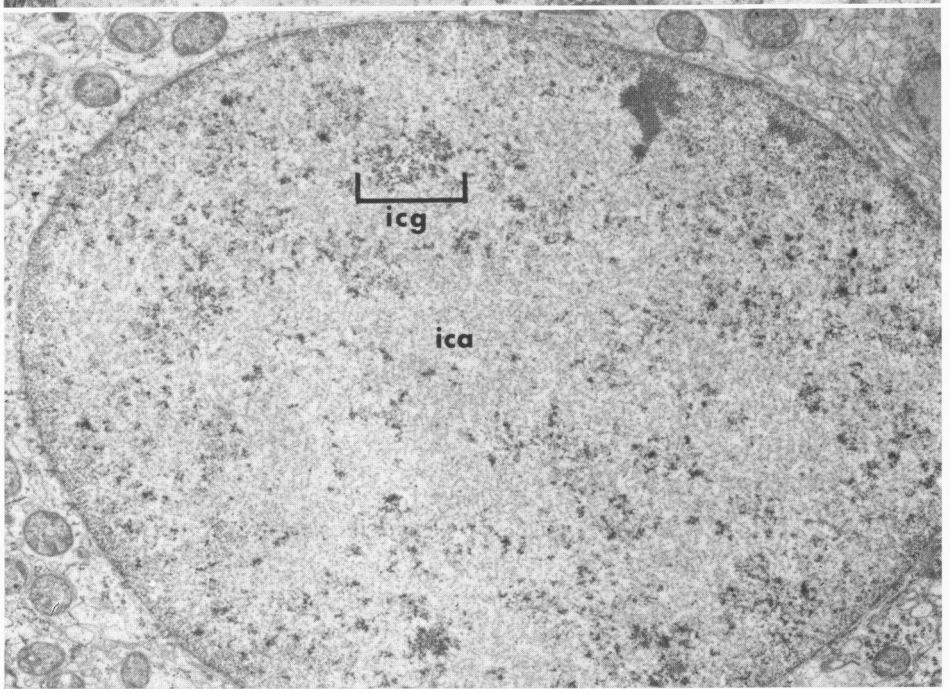

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FIG. 25. The lumen spaces seen by light microscopy prove to be vascular sinusoids, often without a continuous lining. Red cell (RBC); lumen of sinusoid (L). Lead stain. × 10,200.

FIG. 26. In the nuclei of most tumor cells, typical nucleoli are absent or small. The nucleus contains extensive interchromatinic areas (ica) with fibrils, probably DNA, and granules resembling interchromatin granules (icg). Lead stain, × 5,700.