[CANCER RESEARCH 28, 1703–1733, September 1968]

A Comparison of Ultrastructural Changes in Rat Liver Due to Chemical Carcinogens[1]

Donald Svoboda and John Higginson

Department of Pathology, University of Kansas Medical Center, Kansas City, Kansas 66103, and International Agency for Research on Cancer, Lyon, France

SUMMARY

A wide range of nonspecific and reversible ultrastructural responses occur in liver cells following acute and chronic exposure to hepatocarcinogens. Although most changes do not occur to a uniform extent in all cells in acute and chronic experiments, nor do they persist indefinitely after withdrawal of the carcinogen, nucleolar abnormalities and disturbance in the ribosome-ergastoplasm relationship are the most consistent alterations in acute and chronic intervals and in the tumor cells themselves. None of the carcinogens used produced tumors with ultrastructural features sufficiently characteristic to distinguish them from those produced by other carcinogens. Despite the limitations of sampling and methods of electron microscopy, it would appear from a morphologic point of view that, following administration of hepatocarcinogens, nucleolar abnormalities at the level of synthesis and assembly of ribosomal precursors probably are equal in importance to changes in the ribosome-ergastoplasm complex in the cytoplasm.

INTRODUCTION

In studying the role of synergism in liver cancer, a systematic investigation was undertaken to determine to what extent several hepatocarcinogens of different chemical nature produced similar ultrastructural alterations. Previous studies on the sequence of morphologic damage produced by carcinogens in the liver have been confined mainly to the acute stages, and comparatively few reports of the chronic effects of carcinogens on liver ultrastructure are available. Although several reports on the ultrastructure of liver tumors have been published, these deal primarily with lines of standard experimental tumors or occasionally with human tumors, neither group offering itself to sequential morphologic analysis.

While recognizing the variations in the structural, immunologic, and biologic properties of hepatic tumors produced by different or even the same carcinogen, it was considered that a systematic comparison of the acute and chronic ultrastructural changes might be useful. Not only would it assist in distinguishing irreversible cellular responses from epiphenomena but, at the same time, would demonstrate changes which might be common to all carcinogens under investigation (86). Further, although no single, specific, and fundamental biochemical change has been identified which would explain the carcinogenic effect on the same organ of different chemical carcinogens by a common mechanism, a review of the relationship of the morphologic changes to known biochemical changes is desirable. Available studies suggest that not all neoplastic cells arise by single uniform mechanism and support Hieger's (56) earlier statement that there is no single satisfactory theory of carcinogenesis. Recent trends in molecular biology tend increasingly to implicate changes in the nucleoproteins, cell membranes, or cytoplasmic proteins as sites of fundamenal carcinogenic action. The ultimate significance of such changes requires considerable further clarification. The purposes of this paper are: (*a*) to compare the sequence and type of acute and chronic ultrastructural changes in liver cells following several different carcinogens; (*b*) to separate persistent from reversible change at the subcellular level; and (*c*) to emphasize certain ultrastructural features of nuclei in preneoplastic liver cells.

Since several of the carcinogens have been investigated previously by ourselves and others, we have included references to other reports in order to amplify this review.

MATERIALS AND METHODS

In all experiments, inbred male F-344 rats (A. R. Schmidt, Madison, Wisconsin) were used. The animals averaged 170 grams at the beginning of both acute and chronic experiments. All experimental animals were fed our standard purified diet (140) as follows: vitamin-free casein, 16%; vitamin mix, 2.5% (Nutritional Biochemicals); salt mix USP XIV, 4%; sucrose, 62.5%; corn oil, 10%; alphacel, 5%.

Dose and Time Schedules

The dose for each carcinogen and time at which the animals were examined is given in Table 1 for the acute experiments and in Table 2 for the chronic experiments.

Treatment of Tissues

All animals were sacrificed or biopsied under Metofane anaesthesia. In the chronic experiments, animals were chosen at random for biopsy. After laparotomy they were returned to the experimental group although such animals tend to show an increased latent period (38, 105). When an animal was found to have a liver tumor, it was sacrificed and a complete autopsy

[1] Supported in part by USPHS Grants CA-5680 and CA-8055, and by the Kansas Division of The American Cancer Society.

Received February 1, 1968; accepted May 9, 1968.

Table 1

Agent	Dose and route	Time of liver biopsy and sacrifice (hr)	Reference
Aflatoxin B_1	0.45 mg/kg (p.o.)	24, 48, 72[a]	140
Diethylnitrosamine	140 mg/kg (i.p.)	24, 48, 72	
Dimethylnitrosamine	22.5 mg/kg (i.p.)	24, 48, 72	
Ethionine	1 mg/gm (i.p.)	12, 24	
Lasiocarpine	80 mg/kg (i.p.)	1, 4, 6, 8, 12, 24, 48, 72	143
3'-Methyldimethyl-aminoazobenzene[b]	300 mg/kg (p.o.)	24, 48	
Tannic acid	700 mg/kg (s.c.)	1, 3, 6, 12, 18, 24, 48, 72, 120	107
Thioacetamide	60 mg/kg (i.p.)	18, 24, 48	

Schedule of acute experiments.

[a] Two animals were studied at each interval with every agent.

[b] To compare nucleolar alterations, 2 rats were given 2-Methyldimethylaminoazobenzene, 300 mg/kg p.o., and sacrificed at 24 hr.

Table 2

Agent	Number of animals	Dose	Duration of carcinogen (wk.)	Time of biopsy (wk.)	Total number of tumors
Aflatoxin B_1	6	1 ppm	33	2, 6, 10, 12, 14, 18, 20, 24, 35, 37, 39, 40, 52[c]	3
	9	1 ppm followed by 2 ppm	16 17	18, 24, 22, 26[c], 28, 30, 35, 37, 39, 40, 48, 49, 52	9
Diethylnitrosamine	10	0.55 mg/day	12	2, 3, 6, 10, 12, 14, 16[c], 26, 28, 30, 41, 43	8
	15	0.55 mg/day	23	2, 3, 6, 10, 12, 14, 17, 18, 20[c], 21, 23	13
Dimethylnitrosamine	15	0.4 mg/day	24	2, 3, 4, 6, 10, 12, 14, 19, 20, 24, 26, 28[c], 31, 32, 33, 37, 38, 41, 43, 44, 46	7
Ethionine	10	0.25%	12	2, 6, 10, 12, 14, 16, 35, 50	0
	10	0.25%	24	2, 6, 10, 12, 14, 18, 20, 23[c], 26, 28, 30, 33, 35, 39, 45, 48, 50	10
Lasiocarpine[a]	10	240 mg/kg/wk.	20	9, 13, 16, 20	0
3'-Methyldimethylamino-azobenzene	21	0.06%	14	2, 6, 10, 12, 14, 16, 18, 19, 20, 24[c], 28, 29, 40, 43	20
Tannic acid[b]	6	700 mg/kg	3	1, 2, 3	0
Thioacetamide	20	0.032%	12	2, 6, 10, 12, 14	2
	20	0.032%	24	2, 5, 10, 12, 14, 18, 20, 24, 26, 28, 30, 32, 39, 48[c], 52	8

Schedule of chronic experiments.

[a] Ref. 143.

[b] Ref. 107.

[c] Time of appearance of first tumor.

was done. Samples of tumor- and nontumor-bearing liver as well as small hyperplastic nodules (33, 76) were examined by both light and electron microscopy. In several acute experiments with high doses of dimethylnitrosamine, samples of kidney were also taken.

For histology, samples of liver and tumors were fixed in neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Selected blocks were also stained with periodic acid-Schiff technic (with diastase digestion), Feulgen, and methyl green pyronine stains. In acute experiments, oil red O stains for fat were also done.

Electron Microscopic Methods

Samples of liver and tumors were processed for electron microscopic study as previously described (140). In all instances, several blocks of tissue were fixed in *s*-collidine-buffered osmium only. Additional blocks were postfixed in phosphate-buffered glutaraldehyde and some samples were fixed only in buffered glutaraldehyde. After fixation for 2 hr at 0°C, tissues were dehydrated in a graded series of alcohols and propylene oxide, followed by infiltration for 1 hr in a 50:50 propylene oxide:epon mixture. Blocks were embedded in Epon 812 and the resin polymerized at 60°C for 24 hr. Ultrathin sections

were cut with LKB ultramicrotomes using glass knives. They were stained with lead and studied in an RCA 3G electron microscope. For orientation of blocks, tissues prepared for electron microscopy were sectioned at 0.5 to 1.5μ and stained with azure A-bicarbonate for light microscopic examination.

RESULTS

Acute Toxic Experiments

Certain results relative to nucleolar changes have been discussed by us in more detail elsewhere (142).

Light Microscopy. The histologic lesions produced by the agents used here are well known. However, to permit more meaningful evaluation of the ultrastructural changes, the extent of necrosis produced at the respective dose levels is summarized in Table 3.

Electron Microscopy. Since there is considerable evidence that the alterations in the hepatic mesenchymal tissues are largely epiphenomena bearing no necessary relation to neoplasia, discussion is confined largely to the liver cell. The acute ultrastructural changes in the liver cell are summarized in Table 4.

Nucleus. For descriptive convenience the terminology to designate nuclear alterations has been retained, with some elaboration, from a previous report (142). The term "nucleolar capping" has been used to designate a variety of structural abnormalities in the nucleolus resulting from separation of the fibrillar and granular elements, but is not sufficiently discriminative to distinguish the several morphologic types of changes observed. Accordingly, we prefer to describe segregation essentially in terms of the size of the fibrillar area, its purity, and degree of separation from the granular elements.

1. Macrosegregation implies the presence of distinct, relatively large zones, each composed of pure granules or fibrils. Typically, the nucleolus is compact, with reduction of the granular component, and one or more condensed fibrillar zones are situated at the periphery of the rounded, small nucleolus (nucleolar capping). Aflatoxin B_1 (140, Fig. 1), 3'-Me-DAB[2] (Fig. 1), and lasiocarpine (143, Fig. 4) cause typical macrosegregation.

With tannic acid, while there are distinct light and dark zones in a round, compact nucleolus, the dark zones tend to remain within the interior of the nucleolus rather than at the periphery, and the light zones contain a mixture of granules and fibrils (Fig. 2). Furthermore, the separation of granules and fibrils is not so clear-cut as with the former agents.

2. Microsegregation refers to compact condensations of the fibrillar component which are usually disposed as multiple small aggregates throughout the granular component. The degree of separation is not so distinct or so pure as in macrosegregation. Microsegregation was conspicuous following DMN and thioacetamide. The most uniform nucleolar lesion at 24 hours after DMN consisted of partial separation of the fibrillar and granular components and the formation of several dense plaques at the periphery of the nucleolus (Fig. 3). By 48 hours the granular interior was surrounded by stellate aggregates of mixed granules and fibrils (Fig. 4). Similarly, 24 hours after thioacetamide, fibrils were condensed into several compact "knots" situated within the granular component (Fig. 5),

[2] The following abbreviations are used: 3'-Me-DAB, 3'-methyldimethylaminoazobenzene; DMN, dimethylnitrosamine; DEN, diethylnitrosamine.

Table 3

Agent	Number of hours	Nucleus	Cytoplasm	Glycogen	Necrosis	Oval cell and bile duct proliferation	Fat	Cell infiltration
Aflatoxin B_1	24–72		Decreased basophilia (P)	Decreased (P & M)	+ (P)	±		Neutrophils and mononuclear (P)
Diethylnitrosamine	24–72		Acidophilic cells (F)	Decreased (C)	Hemorrhagic ++ (C)			Macrophages, neutrophils (C)
Dimethylnitrosamine	24–72	Enlarged	Vacuoles (M & P)	Decreased (C)	++ (C)			Neutrophils (C)
Ethionine	24	Increased numbers of nucleoli	Slight diffuse vacuolization					
Lasiocarpine	24		Periodic acid-Schiff globules; swelling	Decreased	+ (C)			
3'-Methyldimethyl-aminoazobenzene	24–48		Swelling	Decreased				Mononuclear (C)
Tannic acid	12–72		Decreased basophilia (C)	Decreased	+ (C & F)		+	Neutrophils (C)
Thioacetamide	24–48	Enlarged	Acidophilic cells (C)	Decreased	+ (C)	±		

Light microscopic changes with carcinogens at acute toxic dose levels. P, peripheral zone; M, midzone; C, central zone; F, focal.

Table 4

Carcinogen	Nucleolus	Nucleoplasm	Rough endoplasmic reticulum	Smooth endoplasmic reticulum	Golgi
Aflatoxin B_1	Macrosegregation	Increased interchromatin granules.	Detachment of ribosomes.	Increase ++ Concentric association with glycogen.	
Diethylnitrosamine			Detachment of ribosomes.	Increase +	
Dimethylnitrosamine	Microsegregation		Detachment of ribosomes.	Increase +	
Ethionine	Microsegregation	Increased interchromatin granules.	Detachment of ribosomes.	Increase +	
Lasiocarpine	Macro- and microsegregation	Increased interchromatin granules. "Satellite" granules.	Detachment of ribosomes. Extensive dilatation.	Increase ++	Dilated; numerous small, dense deposits.
3'-Methyldimethylaminoazobenzene	Macrosegregation		Detachment of ribosomes.	Increase ++	
Tannic acid	Macrosegregation	Increased interchromatin granules. Iron-containing inclusions.	Detachment of ribosomes.	Increase ++	Slight dilatation.
Thioacetamide	Increased granular component ++; microsegregation	Increased interchromatin granules.	Detachment of ribosomes; polysomes conspicuous.	Increase ++	

while at 48 hours microsegregation was fully developed (Fig. 6). No significant changes were seen with DEN.

With certain agents (notably aflatoxin B_1, lasiocarpine, tannic acid, and thioacetamide), interchromatin granules appeared increased in number.

The limited number of Kupffer cells examined showed nucleolar changes comparable to those in the parenchymal cells with aflatoxin B_1, lasiocarpine and, to a lesser extent, with 3'-Me-DAB but not with the other carcinogens. The nucleoli of ductular cells showed no consistent changes.

Cytoplasm. These changes are indicated in Table 4. Most agents caused an increase in free ribosomes and dilatation of cisterns of endoplasmic reticulum. Abundant smooth endoplasmic reticulum was prominent in many cells (Fig. 7). Variable degrees of mitochondrial swelling and increased lysosomes were also conspicuous. A slight increase in the number of microbodies was apparent after thioacetamide and DMN.

A notable feature 24 hours after DEN or DMN (and to a lesser extent following 3'-Me-DAB) was the presence of numerous circular coated vesicles measuring 60 mμ, apparently arising from the plasma membrane (Fig. 8). Similar vesicles were also common in the interior of the cell (Fig. 9).

No changes were observed in vascular sinusoids or in portal vessels of the liver following DMN or DEN. In most cells the plasma membranes appeared normal, with the exception of DMN, where interruptions were frequent (Fig. 8).

Chronic Experiments

Light Microscopy. Obvious architectural and cellular change was minimal in the groups treated with aflatoxin B_1 and the nitrosamines. In aflatoxin-treated animals (at both dose levels), there were several scattered foci of small hyperbasophilic cells and occasional groups of large, clear cells lacking glycogen. Cirrhosis, cholangiofibrosis, or proliferation of oval cells was

Table 4 (continued)

Mitochondria	Lysosomes	Microbodies	Glycogen	Fat	Remarks	References
Swelling + (centrolobular) Dense deposits (? calcium) Decrease or loss of matrix granules.	Increase ++		Lost throughout most of lobule, slight centrolobular preservation.	Medium to large globules ++		10, 19, 22, 119, 140
	Increase +		Sharply demarcated centrolobular loss.	Increase +	"Coated" vesicles ++	82
		Moderate increase in number.	Sharply demarcated centrolobular and periportal loss; retained in midzonal areas.	Increase +	"Coated" vesicles ++, interruptions in plasma membranes.	9, 32, 46
						44, 55, 85
Irregular swelling; not uniform in any single cell or from cell to cell.	Increase +++		Uniform loss throughout all zones.	Increase ++	Nuclear changes throughout lobules; cytoplasmic changes primarily centrolobular.	143
Focal mitochondrial swelling.	Increase +		Uniform decrease throughout all zones.	Increase +	"Coated" vesicles	99, 147
Slight swelling +.	Increase +		General loss at 6 hr, restoration in periportal areas by 12 hr.	Increase ++	Focal cytoplasmic necrosis most prominent in centrolobular zones.	107
Dense deposits (? calcium) in occasional cells.	Increase +	Moderate increase in number.	Generally lost throughout lobule except for scattered midzonal cells.	Increase +		42, 145

Early ultrastructural changes in liver cells produced by hepatocarcinogens (up to 72 hours).

not a feature. In rats given DEN there was slight irregularity in cell size at 12 weeks followed by irregular nodular hyperplasia at 16 weeks when the first hepatoma occurred. With DMN there were varying degrees of acidophilic degeneration of centrolobular cells and large hyperchromatic nuclei in some cells.

At 2 weeks rats given 0.25% ethionine showed slight fat accumulation and large, often multiple, eosinophilic nucleoli accompanied, at 14 weeks, by slight oval cell proliferation.

The sequence of histologic changes in rat liver during administration of carcinogenic azo dyes has been well documented (37, 92). In these experiments the typical hyperplastic nodules accompanied by bile duct proliferation were apparent by 14 weeks.

At 2 weeks the centrolobular cells in livers of rats given thioacetamide showed marked enlargement of nucleoli, which progressed peripherally. Oval cell proliferation was apparent at 18 weeks. Thereafter, focal cytoplasmic necrosis, nucleolar enlargement, and slight oval cell proliferation persisted, and ceroid was present in many centrolobular cells.

Reversibility and tumors were studied with respect to the following:

Aflatoxin. Immediately after withdrawal of aflatoxin B_1, hyperbasophilic areas, foci of glycogen-free clear cells, and enlargement of the nucleoli persisted. The first tumor appeared at 52 weeks, and all tumors appeared in livers showing relatively little architectural abnormality. Many tumors were compact, well-differentiated hepatomas or adenohepatomas. In some, prominent circular or sinuous spaces resembling vascular lumens were conspicuous.

DEN. After 12 weeks the livers of rats given DEN showed periportal fatty change with slight megalocytosis and focal atrophy. The first hepatoma occurred at 16 weeks. The tumors were generally poorly differentiated hepatocellular carcinomas

Table 5

Carcinogen	Nucleolus	Nucleoplasm	Rough endoplasmic reticulum	Smooth endoplasmic reticulum	Golgi
Aflatoxin B_1	Microsegregation; slight		Slight dilatation; detachment of ribosomes.	Increased ++	
Diethylnitrosamine			Detachment of ribosomes; polysomes conspicuous.	Increased ++	Dilated, empty vesicles.
Dimethylnitrosamine	Multiple clumped and cord-like condensations of fibrillar component; microsegregation.	Increased interchromatin granules. +	Slight detachment of ribosomes.	Small aggregates of vesicles. ++	Dilated, empty vesicles.
Ethionine	Enlarged; involved both fibrillar and granular components. Occasional nucleoli surrounded by a membrane.	Marked increase in interchromatin granules. ++ Cytoplasmic invaginations. + Increased perichromatin granules. +	Prominent detachment of ribosomes.	Increased ++	Dilated vesicles with dense droplets. ++
Lasiocarpine	Microsegregation in megalocytes.	Increased interchromatin granules. + Cytoplasmic invaginations.	Dilatation; detachment of ribosomes.	Increased ++	Dilated vesicles.
3'-Methyldimethylaminoazobenzene	Occasional nucleoli with condensations of fibrillar component.		Detachment of ribosomes.	Increased ++; close association with glycogen.	Dilated, empty vacuoles.
Tannic acid	Enlargement (rare)	Increased interchromatin granules. Iron-containing inclusions.	Dilatation + detachment of ribosomes.	Increased +	
Thioacetamide	Increased size; granular component; prominent "cavities" in nucleoli.	Increased extent of interchromatic spaces.	Detachment of ribosomes.	Increased ++	Dilated, empty vesicles.

Table 5 (continued)

Mitochondria	Lysosomes	Microbodies	Glycogen	Fat	Remarks	References
Marked elongation of occasional profiles.	+		Patchy loss in scattered foci of enlarged, vacuolated cells.	+	Occasional dilated, bile canaliculi.	18, 88, 140
Swollen (slight).	+		Centrolobular loss.	+		45, 82, 146
Swollen; occasionally ruptured; decreased number and length of cristae, abnormal disposition of cristae; bizarre forms; paucity of matrix granules; interlocking protrusion and evaginations (all changes most marked in periportal zones).	+	Increased in some cells. +	Patchy loss.	++		69, 70, 71
Matrix granules reduced or absent. Large circular dense deposits in matrix.	+		Moderate centrolobular loss.	++		30, 33, 36, 81, 133, 152
Variation in size and shape; scalloping of margins.	++		Decreased.	+		16, 120, 121
	+		Decreased.	Large vacuoles in close association with mitochondria.		66
	+		Decreased.	+		65, 107
Decreased number of matrix granules.	+	Increased in some cells. +	Centrolobular loss; cells lacking glycogen coincided with those having greatest degree of nucleolar enlargement.	++	Annulate lamellae in many cells.	116, 145

Chronic ultrastructural changes produced in liver cells by hepatocarcinogens.

with some areas of adenohepatoma. The tumor cells contrasted markedly with the cytoplasmic basophilia of surrounding nonneoplastic liver cells.

DMN. The first tumor, a poorly differentiated hepatocellular carcinoma, appeared 4 weeks after withdrawal of dimethylnitrosamine. The nonneoplastic liver showed slight megalocytosis and occasional foci of bile duct proliferation which persisted throughout the remainder of the experiments. At 17 weeks after withdrawal, there was uniform centrolobular atrophy which was absent at 20 weeks. The tumors that occurred in this group were hemorrhagic and poorly differentiated.

Ethionine. Four weeks after withdrawal of ethionine, general cytoplasmic basophilia and nucleolar size tended to return to normal, but ill-defined foci of hyperbasophilia still were evident by 6 weeks after withdrawal. Only very slight bile duct proliferation remained at 21 weeks after withdrawal.

3'-Me-DAB. By 4 weeks after withdrawal, diffuse and focal regeneration was prominent and there was increased lobulation, bile duct proliferation, and focal cytoplasmic necrosis. At 24 weeks the first tumor, a hepatocellular carcinoma, was seen. The remaining tumors were trabecular carcinomas, adenocarcinomas, and they had occasional anaplastic or mixed patterns.

Thioacetamide. Two weeks after withdrawal, only a few enlarged nucleoli remained. Few oval cells and small amounts of ceroid were present until 8 weeks, when cytoplasmic basophilia was uniformly restored and most nucleoli appeared normal. There was remoulding of the lobular architecture with long, narrow, parallel cords of liver cells, particularly in midzonal areas.

At 48 weeks the first tumor appeared. Hepatomas were present in 8 out of 20 rats by 52 weeks. The tumors were compact, well-differentiated hepatocellular carcinomas, although some contained cystic spaces.

Electron Microscopy. The most prominent and constant ultrastructural changes in the chronic experiments are listed in Table 5 without reference to the sequence of their development. As in the acute experiments, the ultrastructural alterations in both the nucleus and the cytoplasm (with the exception of mitochondrial changes after DMN) occurred in all zones of the hepatic lobule.

Aflatoxin B_1. In contrast to the acute experiments, the ultrastructural changes in chronic aflatoxin B_1 intoxication were slight at both dose levels and were present in only an occasional cell. Slight microsegregation of the nucleolus was seen. This occurred at 2 weeks and persisted during treatment but reversed from 4 to 6 weeks after withdrawal. Macrosegregation was not present. The main cytoplasmic changes described in Table 5 regressed at 4 to 6 weeks, leaving only hyperplasia of the smooth endoplasmic reticulum.

Tumor cells had few small mitochondria, moderate numbers of microbodies, and short dilated segments of endoplasmic reticulum (Fig. 10). Nucleoli were usually abnormal in their configuration and showed some degree of segregation of constituents. In contrast to the nonneoplastic liver, there were extensive collections of interchromatin granules.

DEN. The nuclei showed no changes, and cytoplasmic changes were minimal apart from ribosomal detachment in many cells. In some cells, vesicles of smooth endoplasmic reticulum were conspicuous. These changes were not constant throughout the organ.

Tumor cells were characterized by abnormal configurations of endoplasmic reticulum. In most cells, extensive concentric circular whorls of smooth endoplasmic reticulum enclosed central collections of lipid droplets. Elsewhere, irregularly disposed, partially granulated cisterns of endoplasmic reticulum coursed amidst small, poorly developed mitochondria and collections of vesicular smooth endoplasmic reticulum (Fig. 11). Close topographic association of glycogen with vesicles of smooth endoplasmic reticulum was a prominent finding. In contrast to acute and chronically damaged liver, nuclei of tumor cells contained dense aggregates of interchromatin granules.

Four weeks after withdrawal of the carcinogen, the only uniform abnormality was the presence of excessive smooth endoplasmic reticulum and free ribosomes (Fig. 12).

DMN. Nuclei of occasional cells showed nucleolar aggregates measuring 100 mμ, forming knot-like condensations of the fibrillar part (microsegregation) (Fig. 13).

In most cells cisterns of endoplasmic reticulum were dilated and small amounts of free ribosomes were present. The most striking changes were in the mitochondria, especially in the peripheral zone. By 2 weeks these organelles had abnormal profiles with decrease in number and length of cristae (Fig. 14). At 4 weeks, numerous swollen bizarre forms were present, and at 6 weeks many swollen mitochondria were identifiable only by their double limiting membranes. The degree of swelling was variable among mitochondria. Interlocking protrusions and invaginations between adjacent profiles were conspicuous (Fig. 15). Occasionally, rupture or loss of one or both membranes was apparent (Fig. 16). The changes persisted during administration of the carcinogen.

By 4 weeks after withdrawal, the most conspicuous abnormality was the increase in smooth endoplasmic reticulum vesicles, and mitochondria returned to normal. In many cells nucleoli showed separation of the fibrillar and granular components (Fig. 17) with a marked preponderance of the latter. Interchromatin granules remained increased and were present in the tumors.

Ethionine. Nucleoli were enlarged due to an increase in both granular and fibrillar constituents, but segregation was not apparent. There was also conspicuous increase in interchromatin granules which often formed compact irregular or branching configurations (Fig. 18); these became more severe after 12 weeks and were accompanied by an increase in perichromatin granules. At 6 to 12 weeks cytoplasmic invaginations into nuclei were common. Nucleoli with membranes were noted occasionally.

A few small areas of focal cytoplasmic necrosis were present. Small, moderately electron-dense droplets were present in the dilated Golgi vesicles. Normal mitochondrial matrix-dense granules were usually reduced or absent. Smooth endoplasmic reticulum was increased.

The nuclear and cytoplasmic changes persisted at 18 weeks and were also seen in the tumors, although mitochondria were reduced in number.

In tumors, cytoplasmic lipid globules were prominent and the endoplasmic reticulum was poorly developed and disposed

in an irregular sparse pattern (Fig. 19). Although some segments of endoplasmic reticulum contained attached ribosomes, free ribosomes appeared equally prominent (Fig. 20). At 2 weeks after withdrawal, mitochondrial profiles were irregular and free ribosomes remained conspicuous (Fig. 21).

At 4 weeks after withdrawal, although some cells still had increased vesicles of smooth endoplasmic reticulum, in many, parallel stratified cisterns of endoplasmic reticulum with attached ribosomes were prominent (Fig. 22). At this interval matrix-dense granules reappeared in mitochondria. In most cells, focal cytoplasmic necrosis was apparent.

3'-Me-DAB. In occasional cells the nucleoli showed slight peripheral microsegregation which persisted until withdrawal of the carcinogen.

At 2 weeks, the liver cells contained several large lipid vacuoles, often in close association with mitochondria. Additional changes were similar to those described by Porter and Bruni (99).

By 4 weeks after withdrawal, most cells were essentially recovered (Fig. 23), apart from focal dilation of cisterns of endoplasmic reticulum and of Golgi vesicles and few small foci of cytoplasmic necrosis. Nuclei, microbodies, and mitochondria were not remarkable.

Tumors were similar to those described after chronic administration of DAB (138).

Thioacetamide. The main change in the nucleus of centrolobular cells was marked enlargement of the nucleoli and increase in the extent of the interchromatinic areas which persisted throughout the experiment. The nucleolar enlargement was due to increase solely in the granular component, the fibrillar portion being inconspicuous or masked in most cells.

At 2 weeks the most prominent cytoplasmic abnormalities consisted of moderate to marked increase in smooth endoplasmic reticulum and detachment of ribosomes. At 6 weeks these changes persisted and were accompanied by several cytoplasmic fat droplets. Focal cytoplasmic necrosis was common, and annulate lamellae were seen in many cells. The number of microbodies appeared increased in some cells. By 14 weeks focal cytoplasmic necrosis had become more extensive.

The first tumor occurred at 48 weeks. Tumor cells characteristically contained abundant smooth endoplasmic reticulum and free ribosomes and comparatively scant cisterns of rough endoplasmic reticulum. Nuclei of tumor cells had large interchromatinic spaces.

Four weeks after withdrawal, most cells continued to have increased amounts of smooth endoplasmic reticulum, while at 6 weeks it was less abundant and cells more nearly resembled the normal (Fig. 24).

Results with lasiocarpine and tannic acid given for shorter periods have been published elsewhere (107, 143).

At no stage in any of the chronic studies were virus-like particles observed in tumors or in nontumorous liver.

DISCUSSION

The present observations confirm the well-known lack of histologic correlation, both quantitatively and qualitatively, between acute and chronic toxic liver damage and later tumor development. In contrast to the severe damage produced by 3'-Me-DAB or thioacetamide, animals treated with aflatoxin or nitrosamines showed relatively little damage in nonneoplastic liver. This lack of correlation supports the view that the primary lesion is parenchymal and that mesenchymal changes are incidental and reverse on withdrawal of the carcinogen. This reversal has also been found in mice treated with a variety of carcinogens where only hepatic cell regeneration was autonomous (106). Farber (35) also concluded that neither zonal necrosis of the liver nor diffuse fibrosis are essential to the neoplastic process. In the present investigation, the chronic doses were not examined for their possible effects at acute intervals, but it is clear that many of the fine structural changes in both acute and chronic experiments are nonspecific and, due to their irregularity in appearance or their reversibility, are not fundamental manifestations of malignant transformation.

Practical and Theoretical Limitations of Current Electron Microscopic Methods in the Study of Carcinogenesis

The following limitations of the present methods require consideration. First, the sampling limitations of electron microscopic technics constitute a serious restriction in distinguishing an ultrastructural equivalent of the wide variety of cellular changes seen by light microscopy. The foci of hyperbasophilic cells prominent in the preneoplastic stages of azo dye carcinogenesis (83) or of vacuolated cells in aflatoxin tumorigenesis are not readily identifiable on gross examination at biopsy or even with light microscopic examination of tissue blocks prepared for electron microscopy.

Further, since only one or two cells may show irreversible changes theoretically characteristic of preneoplasia, it is clear that, with random sampling, a vast number of sections would have to be examined in order to be certain that such cells had not been missed. In the present study, the distinct qualitative difference between tumor and nontumor tissue would indicate that intermediate cells, if they exist, are rare and would be difficult to identify.

Similarly, Kirby has pointed out (62) that a tumor induced by chemical means contains a mixed population of cells with different potentialities indicated by rates of growth upon transplantation and by susceptibility to cytotoxic agents. It is not known whether such differences arise during or after malignant transformation or whether they are already dormant in the normal cells. In either instance it seems doubtful that such differences could be detected by present ultrastructural technics.

Another problem is the lack of sensitivity of ultrastructural methods in detecting phenomena which may be conceived as being essentially nonstructural at present levels of resolution.

Additional considerations related to this problem are implicit in the study of indirect mechanisms of cancer induction (60). Common to all such experiments is a process by which the cells become neoplastic due to alterations in the host rather than to a direct effect of an exogenous carcinogenic stimulus on the cells. It is unlikely that structural changes would be apparent under such conditions.

While viral-induced tumors may contain specific cellular antigens characteristic for all tumors produced by a particular virus (101), chemically induced tumors (with some exceptions

[110]) contain antigens that vary from tumor to tumor suggesting that two different mechanisms exist at the molecular level. Study of the available literature, however, does not reveal consistent morphologic differences between these two broad groups of induced tumors. It should be noted that similar limitations exist in biochemical approaches in which critical changes during the preneoplastic intervals may be diluted by the mass of normal liver (109). In addition, homogenates may contain considerable amounts of nucleotides, enzymes, and other constituents derived from inflammatory cells and necrotic liver cells.

Possible Significance of the Acute and Chronic Changes

In the following discussion, we have attempted to correlate the morphologic lesions and their possible functional significance in relation to the carcinogenic process. Because of the wide spectrum of ultrastructural changes produced, it is not possible to consider in detail the significance of each lesion. Discussion is limited, therefore, to certain of the more important effects with specific reference to changes in the nucleus.

The histologic, carcinogenic, and metabolic lesions produced by ethionine (30, 36, 55), diethylnitrosamine (41, 45, 82, 108, 146), dimethylnitrosamine (6, 8, 9, 21, 29, 31, 32, 46, 68–70, 72, 73), thioacetamide (13, 42, 64, 79, 99, 116, 125, 145), aflatoxin (18, 22–24, 119, 129, 150, 151), tannic acid (65, 107) and lasiocarpine (16, 118, 120, 121, 143) have been the subject of numerous studies. Mikata and Luse (77) have also discussed the changes seen in chronic administration of *N*-2-fluorenyldiacetamide.

In considering the possible metabolic counterparts of the ultrastructural lesions, it is important to differentiate between the toxic, possibly reversible and the irreversible, probably carcinogenic effects of the agents.

Nucleus

Some form of rearrangement of the nucleolar constituents occurred with all the carcinogens shortly after their administration. Macrosegregation was common to aflatoxin B_1 tannic acid, 3′-Me-DAB, and lasiocarpine, while microsegregation occurred with DMN, lasiocarpine, 3′-Me-DAB, and thioacetamide. These lesions have been discussed elsewhere (24, 142). The two types of change are not mutually exclusive, nor necessarily specific for any agent. In certain cases the change is clearly dependent on the dose and time interval. For example, with lasiocarpine, microsegregation at one hour is followed by macrosegregation at 12 hours. In animals treated with 3′-Me-DAB, on the other hand, both changes may be present simultaneously (Fig. 1). With other agents, however, these lesions have not been observed either sequentially or simultaneously. Thus, while it is quite probable that the two changes may reflect only quantitative differences in the degree of nucleolar injury, they may also reflect qualitative differences as well. A relationship of some of the acute, reversible nucleolar alterations in parenchymal cells to carcinogenesis is open to question since, with aflatoxin B_1, lasiocarpine, and 3′-Me-DAB, similar lesions were present in Kupffer cells.

In the chronic experiments typical macrosegregation was not seen during the interval prior to tumor formation, but certain types of microsegregation consisting either of peripheralization or knot-like condensations of the fibrillar components were noted with DMN and to a lesser extent with aflatoxin B_1 and 3′-Me-DAB.

Further, with thioacetamide, the increase in the granular component noted at the acute dose level persisted in chronic experiments and, with ethionine, both the fibrillar and granular components remained increased. The marked increase in the granular component following thioacetamide was also found by Smetana *et al.* (125) and probably represents increase in high molecular weight RNA in the nucleoli (130) or inhibition of migration of RNA from the nucleus and nucleolus to the cytoplasm (64, 136).

Although the main effects of tannic acid on nuclear constituents are not known, aflatoxin B_1, lasiocarpine, ethionine, and DMN all interact with nucleic acids with resultant changes in DNA, RNA, or both. Similarly, certain of the carcinogenic azo dyes bind to DNA, and, because this binding can be inhibited by prefeeding a high riboflavin diet, it was suggested that binding by DNA by an azo dye may be necessary to initiate carcinogenesis (128).

It should be noted, however, that the consequence of carcinogen-DNA binding may not be consistent. For example, aflatoxin B_1, 3′-Me-DAB, and ethionine (135) in acute experiments caused a drop in nuclear RNA content (128) while, in chronic experiments with ethionine, Turner and Reid (148) showed that RNA in purified nuclei was increased up to 50%.

Christie and Le Page (21) concluded that, with DMN treatment, enlarged nuclei became progressively more numerous in rat liver and that they possessed an RNA content proportional to their volume. The observation that DMN, like the pyrrolizidine alkaloids, is mutagenic suggests possible selective nuclear action, but the large nucleus phenomenon has been observed after administration of other hepatotoxic agents that are not mutagenic and is not an uncommon reaction of the liver cell to chronic toxic injury. As with several carcinogens, there would appear to be no definite correlation between the toxic and carcinogenic action of DMN (71).

The increase in interchromatin granules with aflatoxin B_1, lasiocarpine, tannic acid, and thioacetamide in the acute stages and with DMN and ethionine in chronic stages is of interest since, with rare exception (81, 133) little change has been reported in these granules in experimental conditions. Since the original morphologic identification of interchromatin granules (11, 144), their chemical characterization has been extended considerably by Busch *et al.* (19), who showed that these granules comprise a significant part of a complex intranuclear, interchromatinic network probably composed of both RNA and protein. Narayan and Busch (87) have shown that fibrils, which form a major part of the network, are not seen *in situ* with customary methods of fixation and suggested that they were masked by the presence of ribonucleoprotein and deoxyribonucleoprotein. Although the interchromatin particles resemble the ribonucleoprotein particles of the nucleolus in many respects, they are larger, denser, and insensitive to ribonuclease. Accordingly it was suggested that the former are more complex and are probably the site of preferential synthesis of adenine + uridine-rich RNA in contrast to guanine + cytosine-rich nu-

cleolar RNA (131). Another possibility is that the increase in interchromatin granules represents compensatory synthesis of RNA at extranucleolar sites, since many of the hepatocarcinogens in the present study inhibit nucleolar RNA synthesis in acute experiments (unpublished observations). Arguing along similar lines, the dense plaques in nucleoli showing microsegregation may contain RNA and protein (149), since similar structures have been described after actinomycin in neoplastic cells (149) and in nonneoplastic liver cells (117) after ultraviolet radiation (84), antibiotics (124), aflatoxin B_1 (10, 140), lasiocarpine (143), and in animals given thioacetamide followed by actinomycin (123). It is of interest, however, that there was no correlation between the presence of interchromatin granules in chronically injured liver cells and in the tumors.

In summary, it is apparent that while some form of nuclear or nucleolar abnormality is found throughout the chronic stages with all the carcinogens used except DEN, the precise biologic significance of this variety of acute and chronic nuclear changes cannot be stated with certainty. However, preliminary biochemical studies on isolated nucleoli in the acute period indicate decreased nucleolar RNA synthesis and/or concentration with all agents (J. Reddy and D. Svoboda, unpublished observations). At present, studies are being extended to the chronic stages. It has been shown (F. Kume and M. Chiga, personal communication) that the macrosegregation occurring with actinomycin can be temporarily inhibited by pretreatment with thioacetamide. Kume and Chiga suggest that the nucleolus maintains normal morphology despite suppression of RNA synthesis by actinomycin, so long as transport of RNA from nucleolus to cytoplasm is inhibited as by thioacetamide.

Cytoplasm

Endoplasmic Reticulum. Despite variations in the degree of cytoplasmic alterations, there was ribosomal detachment from ergastoplasmic membranes and increased smooth endoplasmic reticulum with all carcinogens in both acute and chronic experiments. These changes appeared early in the chronic studies and persisted after withdrawal of aflatoxin B_1, DEN, DMN, and thioacetamide. With ethionine and 3'-Me-DAB, however, the ribosome-ergastoplasm complex showed generally good recovery by 4 to 6 weeks. Free ribosomes were seen in the tumors produced by all agents. Dilation and proliferation of smooth endoplasmic reticulum and detachment of ribosomes have previously been reported with several carcinogens (19, 32, 44, 46, 82, 99, 116, 133, 140, 145, 147, 152). However, whorls of smooth membranes with or without associated glycogen, are a common response to several types of pathologic stimuli (4, 39, 51–54, 133, 134, 142) and detachment of ribosomes also follows noncarcinogenic agents (7, 58, 126, 127, 132, 141). In most instances ribosomal detachment would appear to be accompanied by diminished protein synthesis (7, 126, 127) or, as with DMN (32), inhibition of amino acid incorporation, lipid accumulation, and glycogen depletion. Baglio and Farber (5) have demonstrated that the appearance of ribosomes in ultrathin sections of liver bear a close correlation with the polysome pattern of isolated ribosomes. Ribosomes attached to membranes appear more active than free ribosomes in protein synthesis as determined in cell-free systems (20, 49, 50). On the other hand, Manganiello and Phillips (75) have shown that, in intact liver slices, free and membrane-bound ribosomes are equally active in protein synthesis. Sabatini *et al.* (115) have suggested that attachment of ribosomes to endoplasmic reticulum is related either to active protein synthesis or to the protein itself being responsible for firm attachment of ribosomes to the membrane.

Glycogen. Close association of glycogen with membranes of endoplasmic reticulum has been noted in ethionine intoxication (50, 133), while depletion of glycogen and encroachment upon glycogen spaces by smooth endoplasmic reticulum was a conspicuous early finding after administration of 3'-Me-DAB (44, 99). In the present experiments changes in the amount of glycogen were only estimated from micrographs of cells from identifiable zones (either clearly periportal or centrolobular). It appeared that glycogen was generally decreased both in acute and chronic experiments, but it is difficult to relate quantitative changes in this labile material to important steps in initiation or promotion of neoplasia.

Mitochondria. No consistent mitochondrial lesion was found with all carcinogens. In acute experiments the main mitochondrial changes consisted of swelling and accumulation of dense matrix deposits, probably calcium, alterations commonly occurring in noncarcinogenic conditions. The large densities in the matrix of mitochondria have been reported previously in chronic ethionine intoxication (152). Minick *et al.* (80) concluded that the intramitochondrial dense bodies were a nonspecific response to cellular injury, apparently derived in part from altered cristae. Somewhat similar results have been obtained in isolated mitochondria incubated with high concentrations of calcium, phosphate, strontium, and barium ions (43, 94).

In acute DMN poisoning, variation in size and confluence of several mitochondria with fragmentation of cristae and irregularity of limiting membranes has been reported (85). Emmelott and Benedetti (32), however, observed no acute alterations in mitochondrial morphology, and mitochondrial and nuclear enzymes had unimpaired activity until several hours after the appearance of defective protein synthesis. Bailie and Christie (6) showed that, within 4 hours after DMN, mitochondrial staining in the central portions of lobules was diminished and that by 12 hours there was a deficiency of DPN-linked respiratory enzymes, with disorganization of oxidative metabolism following between 12 and 20 hours later, when necrosis occurred. It is not clear, therefore, whether the mitochondrial enzyme defects were related to necrosis rather than specifically to DMN.

In contrast to the relatively minimal mitochondrial lesions seen with most carcinogens in the chronic studies, those produced by DMN were severe and have not been reported previously. Mukherjee *et al.* (85) pointed out that liver tissue does not react uniformly to DMN and this may cause a great deal of variation in electron microscopic and biochemical observations. In the present studies, for example, it was clear by light and electron microscopic examination that the enlarged and bizarre forms of mitochondria following DMN were confined to a small number of cells in the immediate periportal area. In preliminary experiments on mitochondria isolated from liver of rats given doses of DMN identical to those used in the present study, there were no detectable abnormalities in oxidative phosphorylation suggesting that any functional changes accompany-

Table 6

Carcinogen	Nucleolus	Nucleoplasm	Rough endoplasmic reticulum	Smooth endoplasmic reticulum	Golgi
Aflatoxin B_1	Dense plaques in nucleoli.	Extensive interchromatinic areas containing interchromatin granules and dispersed fibrils.	Scant; partially surrounding mitochondria.	Vesicles. + Concentric whorls with glycogen between the laminae ("fingerprints"). +	Elongated, flattened vacuoles.
Diethylnitrosamine		Increased interchromatin granules. +	Dilated cisterns partially surrounding mitochondria. Free ribosomes. ++	Vesicles. + Concentric whorls with glycogen between the laminae ("fingerprints"). ++ Occasional myelin figures. Long, flat cisterns with irregular, apparently random, distribution.	
Dimethylnitrosamine		Increased interchromatin granules. +	Scant. Free ribosomes. ++	Vesicles. ++ Myelin figures common.	
Ethionine		Increased interchromatin and perichromatin granules. ++	Scant; partially surrounding mitochondria. Free ribosomes. ++	Vesicles. +	
3'-Methyldimethylaminoazobenzene	Nucleolus enlarged.	Extensive interchromatinic areas. Increased perichromatin granules. + Cytoplasmic invaginations.	Widely dilated cisterns. Free ribosomes. ++	Vesicles. + Annulate lamellae and myelin figures common.	
Thioacetamide	Nucleoli enlarged. ++	Extensive interchromatinic spaces.	Scant. Free ribosomes. ++	Vesicles. +	

ing the pronounced morphologic alterations in mitochondria were masked by the presence of unaltered mitochondria in the midzonal and centrolobular areas. (D. Svoboda, unpublished observations.) That the ultrastructural changes in mitochondria do not bear an essential relationship to carcinogenesis is apparent in that they were absent with all other carcinogens, including DEN. Moreover, tumors occurred earlier and in a greater proportion of animals given DEN than those receiving DMN.

Lysosomes, Microbodies, Golgi Areas, Coated Vesicles. Other than slight dilation and small dense deposits in Golgi vesicles, no prominent abnormality was detected in this organelle. Focal cytoplasmic degradation was more prominent in acute than in chronic studies, probably reflecting a greater degree of cell destruction in acute stages with the higher doses of carcinogens. Persistence of focal cytoplasmic degradation in chronic experiments suggests a low-grade continuous, nonspecific cell injury.

While Allison (2, 3) has emphasized the role of lysosomes as a common mediator of carcinogenesis due to several stimuli, the relatively inconspicuous formation of lysosomes in the present experiments does not suggest a significant role for these organelles in chemical hepatocarcinogenesis.

Microbodies were slightly increased in number in liver cells of animals given thioacetamide and dimethylnitrosamine. Their response to pathologic conditions has received comparatively little attention, although Dalton (25) reported an inverse relationship between microbody population and rate of tumor growth. The biochemical characterization of these organelles has been relatively recent (27). The main enzyme constituents of microbodies are catalase and oxidases probably involved in the production and destruction of hydrogen peroxide. Because of their significant catalase content, it might be expected that they would show some consistent numerical changes in neoplastic cells since depression of hepatic catalase in animals with experimental tumors is well documented (59). Other evidence, however, suggests that only the extraparticulate catalase is decreased (103). Experiments in progress indicate that, in general, tumor cells respond far less than adjacent nontumor cells to the microbody-inducing property of clofibrate-ethyl-α-*p*-chlorophenoxyisobutyrate, a hypolipidemic drug (139).

Coated vesicles have been noted previously in the Golgi re-

Table 6 (continued)

Mitochondria	Lysosomes	Microbodies	Glycogen	Membranes	Remarks
Reduced in size and number with few cristae. Crystalloids in matrix. Few matrix dense granules.	+	Closely associated with partially granulated membranes of endoplasmic reticulum.	Many tumor cells lacked glycogen, cells at periphery of tumors contained glycogen.	Segmental interruptions in external lamina of nuclear envelope and in plasma membranes. ++	Fat droplets. ++ Extensive vascular sinusoids.
Reduced in size and number. Few matrix dense granules.	+		Generalized decrease.	Segmental interruptions in external lamina of nuclear envelope and in plasma membranes. +	
Reduced in size and number.	−	Closely associated with partially granulated membranes of endoplasmic reticulum.	Sparse, often intermixed with vesicles of smooth endoplasmic reticulum.	Segmental interruptions in plasma membranes. +	Fat globules. +
Reduced in size and number. Matrix granules reduced or absent.	+		Absent.	Irregularity in spacing of nuclear pores.	Fat globules. ++
Small and with irregular contours in many cells. Matrix granules absent in most. Many with irregular densities in their matrix.	++		In small hepatocellular tumors (up to 1 cm) 60–75% of cell contained glycogen.		
Small, poorly developed cristae. Matrix granules reduced.	+	Increased in number in some cells.			

Salient ultrastructural features of chemically induced tumors.

The following references also deal with ultrastructural features of hyperplastic nodules and primary or transplanted tumors of rat liver: 11, 14, 17, 25, 28, 33, 57, 67, 76, 77, 89, 91, 138, 140, 142, 149, 151.

gions (15, 34, 90) and in continuity with the endoplasmic reticulum (90, 93, 112) in several cell types (74, 113, 114). Novikoff (90) proposed that they were a means by which specific substances are transported from the endoplasmic reticulum. In normal rat liver (17) and in fetal mouse liver (34), it was suggested that coated vesicles entered multivesicular bodies, and Essner (34) cited evidence favoring transformation of multivesicular bodies into residual bodies rather than into microbodies. The origin of the coated vesicles in present experiments was not clear in all instances, but for the most part they appeared to arise at the plasma membrane (Fig. 8) or in the interior of the cell near the Golgi area. While they were prominent in acute experiments with DEN, DMN, and 3′-Me-DAB, they occur equally frequently 24 hours after administration of actinomycin (unpublished observations). The vesicles may represent accelerated transport of material via specialized portions of the ergastoplasm and plasma membrane (40).

In conclusion, few common morphologic or irreversible cytoplasmic changes are demonstrable at the ultrastructural level with all the carcinogens. Although various degrees of increase in smooth endoplasmic reticulum and detachment of ribosomes persisted throughout all stages, the former might only represent detoxification by enzyme induction and the latter toxic injury. Though both lesions may accompany alterations in protein synthesis, they are obviously not exclusively related to carcinogenesis. For example, La Fontaine and Allard (66) showed that all the cytoplasmic changes produced by the potent carcinogen 3′-Me-DAB were induced by the noncarcinogenic analog 2-Me-DAB. Similarly, in the present experiments, 2-Me-DAB caused acute nucleolar alterations indistinguishable from those due to 3′-Me-DAB.

Reversibility and Tumors

Several ultrastructural studies of experimental hepatic tumors have been reported (14, 25, 57, 67, 138). Brief descriptions of the light (88) and electron microscopic (140) features of tumors induced with aflatoxin B_1 have been reported and an investigation of their biologic behavior is in progress. Though many of these tumors are well differentiated, they grow readily upon subcutaneous and intrahepatic transplantation to inbred recipients (11). In either location, tumors are apparent in 3 to 4 weeks after transplantation. In adrenalectomized hosts,

steroid-induced tryptophan pyrrolase activity in primary and transplanted aflatoxin-induced tumors is only approximately one-fourth that of adjacent nonneoplastic liver (D. Svoboda, unpublished observations). Dalton's comparative study (25) showed that the most rapidly growing tumors gave little evidence of origin from hepatic cells based on ultrastructural characteristics. The rapidly growing tumors had little organized ergastoplasm and simplified Golgi complex, while a slower growing tumor (5123 D) was composed of cells with moderate amounts of ergastoplasm and a Golgi complex with evidence of some secretory activity. Bruni (14) has attempted to classify hepatomas on the basis of their pattern or amount of endoplasmic reticulum.

In the present studies, no carcinogen produced tumors with ultrastructural features sufficiently characteristic to distinguish them from those produced by other carcinogens (Table 6). In general, mitochondria tended to be fewer and smaller than in normal cells; smooth endoplasmic reticulum was increased in many cells, while profiles of granulated endoplasmic reticulum were few, short, and often dilated. Many had segments lacking ribosomes. In contrast to the acute experiments, the nucleolar changes were less prevalent than those in the nucleoplasm. The nuclei in many instances had a relatively "empty" appearance with less than normal amounts of chromatin and extensive interchromatinic spaces. Interchromatin granules were increased with aflatoxin B_1, DEN, DMN, and ethionine.

The most uniform feature of all tumors was the paucity of granular endoplasmic reticulum. In tumors induced by several agents, interruptions in the plasma membrane were conspicuous and clearly require further investigation in view of the possible importance of membrane alterations in neoplasia (1, 42, 89).

Despite the wide variety of lesions produced by each carcinogen, with the exception of nonspecific changes in endoplasmic reticulum and ribosomes, none of the remaining alterations were either specific or consistent in acute, chronic, or tumor stages.

The alterations which persisted in nontumor cells 4 to 8 weeks after withdrawal of the carcinogen are summarized in Table 7. In general, the majority of lesions were reversible in the cells sampled despite the moderate changes outlined in Table 7 (see Figs. 22–24). Comparable recovery after ethionine has been described by Dunn (30), Farber (35), and Wood (152). No salient nuclear alterations could be related with certainty to a respective carcinogen but, instead, appeared to be associated with the stage of toxicity.

Table 7

Carcinogen	Alterations
Aflatoxin	Increased smooth endoplasmic reticulum.
Diethylnitrosamine	Increased smooth endoplasmic reticulum and free ribosomes.
Dimethylnitrosamine	Increased smooth endoplasmic reticulum and interchromatin granules; condensations of the fibrillar component of the nucleolus.
Ethionine	Increased smooth endoplasmic reticulum and interchromatin granules. Focal cytoplasmic degradation in some cells.
3′-Methyldimethylaminoazobenzene	Focal dilatation of smooth endoplasmic reticulum. Focal cytoplasmic degradation.
Thioacetamide	Increased smooth endoplasmic reticulum.

Alterations persisting 4 to 8 weeks after withdrawal of the carcinogen.

Comparison of the Role of the Nucleus to That of the Endoplasmic Reticulum in Carcinogenesis

On the basis of deficient enzyme induction in experimental hepatomas and abnormalities in template stability of messenger RNA in such tumors, Pitot (95, 96) has proposed a defect in structure of the endoplasmic reticulum leading to alterations in the polysome-membrane combination. It was proposed, therefore, that the original site of neoplastic transformation resides in the endoplasmic reticulum and associated polysomes. The observations of Hadjiolov and Dabeva (48) following 3′-Me-DAB are also consistent with this hypothesis. While deficiency of inducible tryptophan pyrrolase activity in tumor cells in liver has been interpreted as a defect residing in the endoplasmic reticulum, derangements in synthesis of ribosomal precursors by the nucleus could be a more proximal cause for derangements in enzyme induction (98). Moreover, the studies of Seidman *et al.* (122) indicate that defective induction of tryptophan pyrrolase may accompany rapid cell division in states other than neoplasia.

That most of the cytoplasmic changes in liver cells following chemical carcinogens are nonspecific reactions to toxicity or manifestations of enzyme induction not necessarily related to initiation of carcinogenesis is supported by the fact that many toxic but noncarcinogenic chemicals cause similar cytoplasmic alterations without affecting nuclear ultrastructure (51, 54). In many instances, however, the nucleus has not been examined or described in detail. Many chemical carcinogens, on the other hand, when given in high doses appear to induce ultrastructural alterations in nuclei prior to those in the cytoplasm. It is difficult, however, to quantitate free ribosomes at first appearance of nuclear alterations because of the variable population of free ribosomes in normal cells. It is conceivable, however, that interruption of genetic transcription from DNA and aberrations in the chemical configuration of messenger RNA or in ribosomal precursors could cause secondary alterations in cytoplasmic ribosomes or in their relation to the endoplasmic reticulum. As pointed out by Magee (69), the reaction of a carcinogen with cytoplasmic protein alone is difficult to accept as the primary cause of tumor growth if one considers that, ultimately, there must be an alteration in the genetic material of the nucleus (but see Ref. 97).

There is considerable biochemical evidence to support the primary importance of the nucleus in carcinogenesis (12, 26, 47, 60). For example, Boyland (12) has proposed 5 mechanisms (depolymerization, precipitation, cross-linking, inhibition of

synthesis, formation of complexes) whereby many types of carcinogens could produce their effects by a reaction with DNA. Similarly, Kitt (63) reported that disturbances in nucleic acid metabolism with consequent modification of genetic structure and function could represent a common basis for the action of several carcinogenic stimuli.

Opposed to the above considerations is the opinion of Pullman (104) who stated that, with the possible exception of alkylating agents, the interaction of carcinogens with protein seemed more directly related to the appearance of cancer than their reaction with nucleic acid, an opinion that coincides with the protein deletion hypothesis (100). Miller and Miller (78) pointed out that alterations of one or more types of messenger could cause a cell to grow independently of normal cellular control mechanisms. If such an altered messenger became self-perpetuating, the neoplastic nature of the parent cell would be transmitted to its progeny without necessary intervention of an abnormal DNA.

In conclusion, the present morphologic observations and related functional problems underscore the importance of Stowell's (137) statement: "We must get down to finer ultrastructural and genic levels before we can reach decisions about nuclear changes in carcinogenesis." It has been stated (28, 91) that there is no specific ultrastructural alteration whereby the malignant cell can be distinguished from the nonmalignant. The present experiments indicate that the same is also true even prior to the appearance of frank neoplasia (28).

CONCLUSIONS

Ultrastructural changes in nuclei and nucleoli during carcinogenesis do not fall into a single specific pattern (143), and many of the acute lesions are, like cytoplasmic alterations, either slight or reversible when the carcinogen is given in chronic, low doses. Nevertheless, the nuclear alterations warrant further study because the biochemical counterparts of the ultrastructural changes must be better understood before final distinctions between essential and nonessential changes can be made. There is substantial evidence that most of the cytoplasmic functional changes related to RNA and protein synthesis are merely results of events initiated in the nucleus. Indeed, it appears that, for most aspects of cell behavior and function, the cytoplasm serves to amplify and elaborate information that originates in the nucleus. Despite the complexity of the nucleus, from the ultrastructural point of view it is imperative that further attempts be directed toward an understanding of the functional counterparts of abnormalities in nuclear structure occurring after exposure to carcinogens.

REFERENCES

1. Abercrombie, M., and Ambrose, D. J. The Surface Properties of Cancer Cells: A Review. Cancer Res., *22:* 525–548, 1962.
2. Allison, A. C. The Possible Role of Lysosomes in Carcinogenesis. Proc. Roy. Soc. Med., *59:* 868–871, 1966.
3. Allison, A. C., and Patton, G. R. Chromosome Damage in Human Diploid Cells Following Activation of Lysosomal Enzymes. Nature, *207:* 1170–1173, 1965.
4. Azarnoff, D., Grady, H., and Svoboda, D. The Effect of DDD on Barbiturate and Steroid-Induced Hypnosis in the Dog and Rat. Biochem. Pharmacol., *15:* 1985–1993, 1966.
5. Baglio, C. M., and Farber, E. Correspondence Between Ribosome Aggregation Patterns in Rat Liver Homogenates and in Electron Micrographs Following Administration of Ethionine. J. Mol. Biol., *12:* 466–467, 1965.
6. Bailie, M., and Christie, G. Acute Toxic Action of Dimethylnitrosamine on Liver Cells. Biochem. J., *72:* 473–479, 1959.
7. Barker, E., Smuckler, E., and Benditt, E. Effects of Thioacetamide and Yellow Phosphorus Poisoning on Protein Synthesis *in Vivo*. Lab. Invest., *12:* 955–960, 1963.
8. Barnes, J. M., and Magee, P. N. Some Toxic Properties of Dimethylnitrosamine. Brit. J. Ind. Med., *11:* 167–174, 1954.
9. Benedetti, E. L., and Emmelot, P. Effect of Dimethylnitrosamine on the Endoplasmic Reticulum of Rat Liver Cells. Lab. Invest., *15:* 209–216, 1966.
10. Bernhard, W., Fraysinnet, C., Lafarge, C., and Le Breton, E. Lésions Nucléolaires Précoces Provoquées par l'Aflatoxine dans les Cellules Hépatiques du Rat. Compt. Rend., *261:* 1785–1788, 1965.
11. Bernhard, W., and Granboulan, N. The Fine Structure of the Cancer Cell Nucleus. *In:* H. Busch (ed.), Experimental Cell Research Suppl. 9, pp. 19–53. New York: Academic Press, 1968.
12. Boyland, E. Different Types of Carcinogens and Their Possible Modes of Action: A Review. Cancer Res., *12:* 77–84, 1952.
13. Brock, N. Druckrey, H., and Hamperl, H. Die Erzeugung von Leberkrebs durch den Farbstoff 4-Dimethylaminoazobenzol. Z. Krebsforsch., *50:* 431–456, 1940.
14. Bruni, C. Observations on the Fine Structure of Slow and Fast-Growing Rat Hepatomas. *In:* S. S. Breese, Jr. (ed.), Electron Microscopy, pp. PP 13. New York: Academic Press, 1962.
15. Bruni, C., and Porter, K. R. The Fine Structure of the Parenchymal Cell of the Normal Rat Liver. Am. J. Pathol., *66:* 691–755, 1965.
16. Bull, L. B., and Dick, A. T. The Chronic Pathological Effects on the Liver of the Rat of the Pyrrolizidine Alkaloids, Heliotrine, Lasiocarpine and Their N-Oxides. J. Pathol. Bacteriol., *78:* 483–502, 1959.
17. Busch, H., Byvoet, P., and Smetana, K. The Nucleolus of the Cancer Cell: A Review. Cancer Res., *23:* 313–339, 1963.
18. Butler, W. H. Liver Injury and Aflatoxin. *In:* G. N. Wogan (ed.) Mycotoxins in Foodstuffs, pp. 175–186. Cambridge: Massachusetts Institute of Technology Press, 1965.
19. Butler, W. H. Early Hepatic Parenchmal Changes Induced in the Rat by Aflatoxin B_1. Am. J. Pathol., *49:* 113–128, 1966.
20. Campbell, P. N., Cooper, C., and Hicks, M. Studies on the Role of the Morphological Constituents of the Microsome Fraction from Rat Liver in Protein Synthesis. Biochem. J., *92:* 225–234, 1964.
21. Christie, G. S., and LePage, R. N. Enlargement of Liver Cell Nuclei: Effect of Dimethylnitrosamine on Size and Desoxyribosenucleic Acid Content. Lab. Invest., *10:* 729–743, 1961.
22. Clifford, J. I., and Rees, K. R. Aflatoxin: A Site of Action in the Rat Liver Cell. Nature, *209:* 312–313, 1966.
23. Clifford, J. I., and Rees, K. R. The Action of Aflatoxin B_1 on the Rat Liver. Biochem. J., *102:* 65–75, 1967.
24. Clifford, J. I., Rees, K. R., and Stevens, E. M. The Effect of

the Aflatoxin B_1, G_1 and G_2 on Protein and Nucleic Acid Synthesis in Rat Liver. Biochem. J., *103:* 258–261, 1967.
25. Dalton, A. An Electron Microscopical Study of a Series of Chemically Induced Hepatomas. *In:* P. Emmelot and O. Mühlbock (eds.), Cellular Control Mechanisms and Cancer, pp. 211–225. New York: Elsevier Publishing Co., 1964.
26. Darlington, C. The Plasmagene Theory of the Origin of Cancer. Brit. J. Cancer, *2:* 118–126, 1948.
27. De Duve, C., Beaufay, H., Jacques, P., Rachman-Li, Y., Sellinger, D., Wattiaux, R., and DeConinck, S. Intracellular Localization of Catalase and of Some Oxidases in Rat Liver. Biochim. Biophys. Acta, *40:* 186–187, 1960.
28. de Harven, E. Electron Microscopy of Cancer Cells: A Review. Med. Clin. N. Am., *50:* 887–900, 1966.
29. Druckrey, M., Preussmann, R., Ivankovic, S., and Schmäl, D. Organotrope Carcinogene Wirkungen bei 65 Verschiedenen N-Nitroso-Verbindungen an BD-Ratten. Z. Krebsforsch., *69:* 103–201, 1967.
30. Dunn, W. L. Ethionine Carcinogenesis in the Rat. J. Pathol. Bacteriol., *89:* 513–524, 1965.
31. Dutton, A. H., and Heath, D. F. Demethylation of Dimethylnitrosamine in Rats and Mice. Nature, *178:* 644, 1956.
32. Emmelot, P., and Benedetti, E. L. Changes in the Fine Structure of Rat Liver Cells Brought About by Dimethylnitrosamine. J. Biophys. Biochem. Cytol., *7:* 393–396, 1960.
33. Epstein, S., Ito, N., Merkow, L., and Farber, E. Cellular Analysis of Liver Carcinogenesis: The Induction of Large Hyperplastic Nodules in the Liver with 2-Fluorenylacetamide or Ethionine and Some Aspects of Their Morphology and Glycogen Metabolism. Cancer Res., *27:* 1702–1711, 1967.
34. Essner, E. Endoplasmic Reticulum and the Origin of Microbodies in Fetal Mouse Liver. Lab. Invest., *17:* 71–87, 1967.
35. Farber, E. Similarity in the Sequence of Early Histological Changes Induced in the Liver of the Rat by Ethionine, 2-Acetylaminofluorene, and 3'-Methyl-4-Dimethylaminoazobenzene. Cancer Res., *16:* 142–148, 1956.
36. Farber, E. Ethionine Carcinogenesis. *In:* A. Haddow and S. Weinhouse (eds.), Advances in Cancer Research, Vol. 7, pp. 383–474. New York: Academic Press, 1963.
37. Firminger, H. I., and Mulay, A. S. Histochemical and Morphological Differentiation of Induced Tumors of the Liver in Rats. J. Natl. Cancer Inst., *13:* 19–33, 1952.
38. Firminger, H. I., and Reuber, M. D. Influence of Adrenocortical Androgenic and Anabolic Hormones on the Development of Carcinoma and Cirrhosis of the Liver in AXC Rats Fed *N*-2-Fluorenyldiacetamide. J. Natl. Cancer Inst., *27:* 559–595, 1961.
39. Fouts, J. Factors Affecting Hepatic Microsomal Enzyme Systems Involved in Drug Metabolism. *In:* G. Weber (ed.), Advances in Enzyme Regulation, Vol. 1, pp. 225–233. Oxford: Pergamon Press, Ltd., 1963.
40. Friend, D. S., and Farquhar, M. G. Functions of Coated Vesicles During Protein Absorption in the Rat Vas Deferens. J. Cell Biol., *35:* 357–376, 1967.
41. Fritz-Niggli, H., Nickel, E., and Meier, D. Die Nichtbeeinflussbarbeit der Oxydativen Phophorylierung in Mitochondrien der Rattenleber durch Verfutterung der Cancerogenen Substanzen Diäthylnitrosamin und 4-Dimethylaminoazobenzol. Naturwissenschaften, *52:* 472–473, 1965.
42. Gallagher, C., Gupta, D., Judah, J., and Rees, K. Biochemical Changes in Liver in Acute Thioacetamide Intoxication. J. Pathol. Bacteriol., *72:* 193–201, 1956.
43. Greenawalt, J. W., Rossi, C. S., and Lehninger, A. L. Effect of Active Accumulation of Calcium and Phosphate Ions on the Structure of Rat Liver Mitochondria. J. Cell Biol., *23:* 21–38, 1964.
44. Grisham, J. W. Early Changes in the Fine Structure of the Hepatic Cell in Ethionine-Fed Rats. Federation Proc., *19:* 186, 1960.
45. Grundmann, E., and Sieburg, H. Die Histogenese und Cytogenese des Lebercarcinoms der Ratte durch Diäethylnitrosamin im Lichtmikroskopischen Bild. Beitr. Pathol. Anat., *126:* 57–90, 1962.
46. 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. Natl. Cancer Inst., *30:* 1045–1075, 1963.
47. Haddow, A. Transformation of Cells and Viruses. Nature, *154:* 194–198, 1944.
48. Hadjiolov, A., and Dabeva, M. Loss of Tryptophane Pyrrolase Co-Factor from Liver Cytoplasmic Structures of 3'-Methyl-4-dimethylaminoazobenzene Fed Rats. Experientia, *17:* 452–453, 1961.
49. Hallinan, T. H., and Munro, H. N. Comparison of Amino Acid Incorporation and Ribonucleic Acid Turnover in Rat Liver Submicrosomal Fractions. Biochem. Biophys. Acta, *80:* 166–168, 1964.
50. Henshaw, E. C., Bojarski, T. B., and Hiatt, H. H. Protein Synthesis by Free and Bound Rat Liver Ribosomes. J. Mol. Biol., *7:* 122–129, 1963.
51. Herdson, P. B., Garvin, P. J., and Jennings, R. B. Biological Changes in Rat Liver Induced by 3-Allyl-5-isobutyl-2-thiohydantoin. J. Cell Biol., *19:* 33A, 1963.
52. Herdson, P. B., Garvin, P. J., and Jennings, R. B. Reversible Biological and Fine Structural Changes Produced in Rat Liver by a Thiohydantoin Compound. Lab. Invest., *13:* 1014–1031, 1964.
53. Herdson, P. B., Garvin, P. J., and Jennings, R. B. Fine Structural Changes in Rat Liver Induced by Phenobarbital. Lab. Invest., *13:* 1032–1037, 1964.
54. Herdson, P. B., and Kaltenbach, J. P. Electron Microscope Studies on Enzyme Activity and the Isolation of Thiohydantoin-Induced Myelin Figures in Rat Liver. J. Cell. Biol., *25:* 485–493, 1965.
55. Herman, L., Eber, L., annd Fitzgerald, P. Liver Cell Degeneration with Ethionine Administration: *In:* S. S. Breese Jr. (ed.), Electron Microscopy, Vol. 2, pp. VV 6. New York: Academic Press, 1962.
56. Hieger, I. Theories of Carcinogenesis. Carcinogenesis, pp. 9–11. New York: Academic Press, 1961.
57. Hruban, Z., Swift, H., and Rechcigl, M. Fine Structure of Transplantable Hepatomas of the Rat. J. Natl. Cancer Inst., *35:* 459–473, 1965.
58. Jézéquel, A. M. Les Effets de l'Intoxication Aiquë au Phosphore sur le Foie de Rat. Étude au Microscope Electronique. Ann. Anat. Pathol., *3:* 512–537, 1958.
59. Kampschmidt, R. F. Mechanism of Liver Catalase Depression in Tumor-Bearing Animals: A Review. Cancer Res., *25:* 34–45, 1965.
60. Kaplan, H. S. Some Implications of Indirect Induction Mechanisms in Carcinogenesis: A Review. Cancer Res., *19:* 791–803, 1959.
61. Kinosita, R. Studies on the Cancerogenic Chemical Substances. Trans. Japan Pathol. Soc., *27:* 665–727, 1937.
62. Kirby, K. S. Nucleic Acids of Cancer. *In:* F. Homburger

(ed.), Progress in Experimental Tumor Research, Vol. 2, pp. 291–331. New York: Verlag S. Karger, 1961.
63. Kitt, F. Nucleic Acid Synthesis in Neoplastic Cell and Impact of Nuclear Changes on the Biochemistry of Tumor Tissue: A Review. Cancer Res., *20:* 1121–1148, 1960.
64. Kizer, D., Shirley, B., Cox, B., and Howell, B. Effect of Thioacetamide on Adenylic Acid Deaminase Activity and Nuclear Ribonucleic Acid Metabolism in Rat Liver. Cancer Res., *25:* 596–603, 1965.
65. Korpassy, B. Tannins as Hepatic Carcinogens. *In:* F. Homburger (ed.), Progress in Experimental Tumor Research, Vol. 2, pp. 245–290. New York: Hafner Publishing Company, 1961.
66. LaFontaine, J. G., and Allard, C. A Light and Electron Microscope Study of the Morphological Changes Induced in Rat Liver Cells by Azo Dye 2-Me-DAB. J. Cell. Biol., *22:* 143–172, 1964.
67. Ma, M. H., and Webber, A. J. Fine Structure of Liver Tumors Induced in the Rat by 3'-Methyl-4-dimethylaminoazobenzene. Cancer Res., *26:* 935–946, 1966.
68. Magee, P. N. The Metabolism of Dimethylnitrosamine. Biochem. J., *64:* 676–686, 1956.
69. Magee, P. N. Cellular Injuries and Chemical Carcinogenesis by N-Nitroso Compounds. *In:* R. Raven (ed.), Cancer Progress, pp. 56–66. London: Butterworth & Co., Ltd., 1963.
70. Magee, P. N., and Barnes, J. The Production of Malignant Primary Hepatic Tumors in the Rat by Feeding Diethylnitrosamine. Brit. J. Cancer, *10:* 114–122, 1956.
71. Magee, P. N., and Barnes, J. N. Carcinogenic Nitroso Compounds. *In:* A. Haddow and S. Weinhouse (eds.), Advances in Cancer Research, Vol. 10, pp. 123–246. New York: Academic Press, 1967.
72. Magee, P. N., and Schoental, R. Carcinogenesis by Nitroso Compounds. Brit. Med. Bull., *20:* 102–106, 1964.
73. Magee, P., and Vanderkar, M. Toxic Liver Injury, Biochem. J., *70:* 600–611, 1958.
74. Maillard, M. Origine des Grains de Sécrétion dans les Cellules de l'Antehypophyse Embryonnaire du Rae; Rôle de l'Appareil de Golgi. J. Microscopie, *2:* 81–94, 1963.
75. Manganiello, V., and Phillips, A. The Relationship between Ribosomes and the Endoplasmic Reticulum during Protein Synthesis. J. Biol. Chem., *240:* 3951–3959, 1965.
76. Merkow, L. P., Epstein, S. M., Caito, B., and Bartus, B. The Cellular Analysis of Liver Carcinogenesis: Ultrastructural Alterations within Hyperplastic Liver Nodules Induced by 2-Fluorenylacetamide. Cancer Res., *27:* 1712–1721, 1967.
77. Mikata, A., and Luse, S. Ultrastructural Changes in the Rat Liver Produced by N-2-Fluorenyldiacetamide. Am. J. Pathol., *44:* 455–479, 1964.
78. Miller, E. C., and Miller, J. A. Mechanisms of Chemical Carcinogenesis. Nature of Proximate Carcinogens and Interactions with Macromolecules. Pharmacol. Rev., *18:* 805–838, 1966.
79. Miller, J. A., and Miller, E. C. The Carcinogenic Aminoazo Dyes. *In:* J. P. Greenstein and A. Haddow (eds.), Advances in Cancer Research, Vol. 1, pp. 340–396. New York: Academic Press, 1953.
80. Minick, O. T., Kent, G., Orfei, E., and Volini, F. Non-membrane Enclosed Intramitochondrial Dense Bodies. Exptl. Mol. Pathol., *4:* 311–319, 1965.
81. Miyai, K., and Steiner, J. Fine Structure of Interphase Liver Cell Nuclei in Subacute Ethionine Intoxication. Exptl. Mol. Pathol., *4:* 525–566, 1965.
82. Mölbert, E., Hill, K., and Buchner, E. Die Kanzerisierung der Leberparenchymzelle durch Diäthylnitrosamin im Elektronenmikroskopischen Bild. Beitr. Pathol. Anat., *126:* 218–242, 1962.
83. Molnar, F., and Daoust, R. Nucleocytoplasmic Ratios in Different Populations of Rat Liver Parenchymal Cells during Azo Dye Carcinogenesis. Cancer Res., *25:* 1213–1218, 1965.
84. Montgomery, P. O'B., Reynolds, R., and Cook, J. Nucleolar "Caps" Induced by Flying Spot Ultraviolet Nuclear Irradiation. Am. J. Pathol., *49:* 555–567, 1966.
85. Mukherjee, T., Gustafsson, R., Afzelius, B., and Arrhenius, E. Effects of Carcinogenic Amines on Amino Acid Incorporation by Liver Systems. II. A Morphological and Biochemical Study on the Effect of Dimethylnitrosamine. Cancer Res., *23:* 944–953, 1963.
86. Nakahara, W. Critique of Carcinogenic Mechanism. *In:* Homburger (ed.), Progress in Experimental Tumor Research, Vol. 2, pp. 158–202. New York: Verlag S. Karger, 1961.
87. Narayan, K., Steele, W., Smetana, K., and Busch, H. Ultrastructural Aspects of the Ribonucleoprotein Network in Nuclei of Walker Tumor and Rat Liver. Exptl. Cell Res., *46:* 65–77, 1967.
88. Newberne, P. M. Carcinogenicity of Aflatoxin-Contaminated Peanut Meals. *In:* G. N. Wogan (ed.), Mycotoxins in Foodstuffs, pp. 187–208. Cambridge, Massachusetts: M. I. T. Press, 1965.
89. Novikoff, A. B., and Biempica, L. Cytochemical and Electron Microscopic Examination of Morris 5123 and Reuber H-35 Hepatomas after Several Years of Transplantation. *In:* T. Yoshida (ed.), Biological and Biochemical Evaluation of Malignancy in Experimental Hepatomas: Gann Monograph 1, pp. 65–87, Tokyo: Japanese Foundation for Cancer Research and the Japanese Cancer Association, 1965.
90. Novikoff, A. B., and Shin, W. Y. The Endoplasmic Reticulum in the Golgi Zone and Its Relations to Microbodies, Golgi Apparatus and Autophagic Vacuoles in Rat Liver Cells. J. Microscopie, *3:* 187–206, 1964.
91. Oberling, Ch., and Bernhard, W. The Morphology of the Cancer Cells. *In:* J. Brachet and A. E. Mirsky (eds.), The Cell, Vol. 5, pp. 405–496. New York: Academic Press, 1961.
92. Opie, E. L. The Pathogenesis of Tumors of the Liver Produced by Butter Yellow. J. Exptl. Med., *80:* 231–246, 1944.
93. Palay, S. L. Alveolate Vesicles in Purkinje Cells of the Rat's Cerebellum. J. Cell Biol., *19:* 89A, 1963.
94. Peachey, L. D. Electron Microscopic Observations on the Accumulation of Divalent Cations in Intramitochondrial Granules. J. Cell Biol., *20:* 95–111, 1964.
95. Pitot, H. C. Molecular Pathogenesis of Experimental Liver Cancer. Federation Proc., *21:* 1124–1129, 1964.
96. Pitot, H. C. Some Biochemical Aspects of Malignancy. Ann. Rev. Biochem., *35:* 335–368, 1966.
97. Pitot, H. C., and Heidelberger, C. Metabolic Regulatory Circuits and Carcinogenesis. Cancer Res., *23:* 1694–1700, 1963.
98. Pitot, H. C., Peraino, C., Lamar, Jr., C., and Kennan, A. L. Template Stability of Some Enzymes in Rat Liver and Hepatoma. Proc. Natl. Acad. Sci. U.S., *54:* 845–851, 1965.
99. Porter, K. R., and Bruni, C. An Electron Microscope Study of the Early Effects of 3'-Me-DAB on Rat Liver Cells. Cancer Res., *19:* 997–1009, 1959.
100. Potter, V. R. Transplantable Animal Cancer, the Primary Standard. Cancer Res., *21:* 1331–1333, 1961.
101. Prehn, R. T. A Clonal Selection Theory of Chemical Carcinogenesis. J. Natl. Cancer Inst., *32:* 1–17, 1964.

102. Price, J. M., Harman, J. W., Miller, E. C., and Miller, J. A. Progressive Microscopic Alterations in the Livers of Rats Fed the Hepatic Carcinogens 3'-Methyl-4-Dimethylaminoazobenzene and 4-Fluoro-4-Dimethylaminoazobenzene. Cancer Res., *12:* 192–200, 1952.
103. Price, V. E., Sterling, W. R., Tarantola, V. A., Hartley, Jr., R. W., and Rechcigl, Jr., M. The Kinetics of Catalase Synthesis and Destruction *in vivo*. J. Biol. Chem., *237:* 3468–3475, 1962.
104. Pullman, B. J. Electronic Aspects of the Interactions Between the Carcinogens and Possible Cellular Sites of Their Activity. J. Cellular Comp. Physiol., *64:* 91–110, 1964.
105. Puron, R., and Firminger, H. I. Protection Against Induced Cirrhosis and Hepatocellular Carcinoma in Rats by Chloramphenicol. J. Natl. Cancer Inst., *35:* 39–37, 1965.
106. Quinn, P. S., and Higginson, J. Reversible and Irreversible Changes in Experimental Cirrhosis. Am. J. Pathol., *47:* 353–369, 1965.
107. Racela, A., Grady, H., and Svoboda, D. Ultrastructural Nuclear Changes Due to Tannic Acid. Cancer Res., *27:* 1658–1671, 1967.
108. Rapp, H., Carleton, J., Cristler, C., and Nadel, E. Induction of Malignant Tumors in the Rabbit by Oral Administration of Diethylnitrosamine. J. Natl. Cancer Inst., *34:* 453–458, 1965.
109. Reid, E. Significant Biochemical Effects of Hepatocarcinogens in the Rat: A Review. Cancer Res., *22:* 398–430, 1962.
110. Reiner, J., and Southam, C. M. Evidence of Common Antigenic Properties in Chemically Induced Sarcomas of Mice. Cancer Res., *27:* 1243–1247, 1967.
111. Reuber, M. Histopathology of Transplantable Hepatic Carcinomas Induced by Chemical Carcinogens in Rats: *In:* T. Yoshida (ed.), Biological and Biochemical Evaluation of Malignancy in Experimental Hepatomas, Gann Monograph 1, pp. 43–54, Tokyo: The Japanese Foundation for Cancer Research and the Japanese Cancer Association, 1965.
112. Rosenbluth, J., and Wissig, S. L. The Uptake of Ferritin by Toad Spinal Ganglian Cells. J. Cell Biol., *19:* 91A, 1963.
113. Roth, T. F., and Porter, K. R. Specialized Sites on the Cell Surface for Protein Uptake. *In:* S. S. Breese, Jr. (ed.), Electron Microscopy, pp. LL 4. New York: Academic Press, 1962.
114. Rouiller, C., and Jézéquel, A. M. Electron Microscopy of the Liver. *In:* C. Rouiller and A. M. Jézéquel (eds.), The Liver, Morphology, Biochemistry, Physiology, Vol. 1, pp. 195–264. New York: Academic Press, 1963.
115. Sabatini, D. D., Tashiro, Y., and Palade, G. E. On the Attachment of Ribosomes to Microsomal Membranes. J. Mol. Biol., *19:* 503–524, 1966.
116. Salomon, J. Modifications des Cellules du Parenchyme Hépatique du Rat sous l'Effet de la Thioacetamide. Étude au Microscope Electronique des Lésions Observées á la Phase Tardive d'une Intoxication Chronique. J. Ultrastruct. Res., *7:* 293–307, 1962.
117. Schoefl, G. The Effects of Actinomycin on the Fine Structure of the Nucleolus. J. Ultrastruct. Res., *10:* 224–243, 1964.
118. Schoental, R. Pyrrolizidine (Senecio) Alkaloids and Their Hepatotoxic Action. Biochem. J., *88:* 57, 1963.
119. Schoental, R. Aflatoxins. Ann. Rev. Pharmacol., *7:* 343–356, 1967.
120. Schoental, R., and Magee, P. N. Chronic Liver Changes in Rats after a Single Dose of Lasiocarpine, a Pyrrolizidine (Senecio) Alkaloid. J. Pathol. Bacteriol., *74:* 305–319, 1957.
121. Schoental, R., and Magee, P. N. Further Observations on the Subacute and Chronic Liver Changes in Rats after a Single Dose of Various Pyrrolizidine (Senecio) Alkaloids. J. Pathol. Bacteriol., *78:* 471–482, 1959.
122. Seidman, I. Hormonal and Substrate Induction of Tryptophan Pyrrolase in Regenerating Rat Liver. Cancer Res., *27:* 1620–1925, 1967.
123. Shankar, N., Steele, W., and Busch, H. Evidence that the Granular and Fibrillar Components of Nucleoli Contain 28 and 6S RNA Respectively. Exptl. Cell. Res., *43:* 483–492, 1966.
124. Simard, R., and Bernhard, W. Le Phénomène de la Segregation Nucleolaire: Specificite d'Action de Certains Antimetabolites. Intern. J. Cancer, *1:* 463–479, 1966.
125. Smetana, K., Narayan, K. S., and Busch, H. Quantitative Analysis of Ultrastructural Components of Nucleoli of the Walker Tumor and Liver. Cancer Res., *26:* 786–796, 1966.
126. Smuckler, E., Iseri, O., and Benditt, E. An Intracellular Defect in Protein Synthesis Induced by Carbon Tetrachloride. J. Exptl. Med., *116:* 55–72, 1962.
127. Smuckler, E. A., Iseri, O. A., and Benditt, E. P. Studies on Carbon Tetrachloride Intoxication. I. The Effect of Carbon Tetrachloride on Incorporation of Labeled Amino Acids Into Plasma Proteins. Biochem. Biophys. Res. Commun., *5:* 270–275, 1961.
128. Sporn, M. B., and Dingman, C. W. Studies on Chromatin. II. Effects of Carcinogens and Hormones on Rat Liver Chromatin. Cancer Res., *26:* 2488–2495, 1966.
129. Sporn, M. B., Dingman, C. W., and Phelps, H. L. Aflatoxin B_1: Binding to DNA *in vitro* and Alteration of RNA Metabolism *in vivo*. Science, *151:* 1539–1541, 1966.
130. Steele, W. J., and Busch, H. Increased Content of High Molecular Weight RNA Fractions in Nuclei and Nucleoli of Livers of Thioacetamide-Treated Rats. Biochem. Biophys. Acta, *119:* 501–509, 1966.
131. Steele, W., and Busch, H. Studies on the Ribonucleic Acid Components of the Nuclear Ribonucleoprotein Network. Biochim. Biophys. Acta, *129:* 54–67, 1966.
132. Steiner, J. W., and Baglio, C. M. Electron Microscopy of the Cytoplasm of Parenchymal Liver Cells in Alpha-naphthylisothiocyanate-Induced Cirrhosis. Lab. Invest., *12:* 765–790, 1963.
133. Steiner, J. Miyai, K., and Phillips, M. Electron Microscopy of Membrane-Particle Arrays in Liver Cell of Ethionine-Intoxicated Rats. Am. J. Pathol., *44:* 169–213, 1964.
134. Stenger, R. J. Regenerative Nodules in Carbon Tetrachloride-Induced Cirrhosis. A Light and Electron Microscopic Study of Lamellar Structures Encountered Therein. Am. J. Pathol., *44:* 31A, 1964.
135. Stewart, G., and Farber, E. Comparative Effects of Ethionine and Actinomycin upon Rat Liver RNA Metabolism. Federation Proc., *25:* 646, 1966.
136. Stöcher, E., and Yokoyama, T. Autoradiographische Studien zum Einfluss von Thioacetamid auf die RNS-Migration and DNS-Synthese Verschiedener Parenchymatöser Zellen der Ratte. Beitr. Pathol. Anat., *134:* 1–28, 1966.
137. Stowell, R. E. Summary of Informal Discussion. Exptl. Cell Res. Suppl., *9:* 107–110, 1963.
138. Svoboda, D. J. Fine Structure of Hepatomas Induced in Rats with p-Dimethylaminoazobenzene. J. Natl. Cancer Inst., *33:* 315–339, 1964.
139. Svoboda, D. J., and Azarnoff, D. L. Response of Hepatic Microbodies to a Hypolipidemic Agent, Ethyl Chlorophenoxyisobutyrate (CPIB). J. Cell Biol., *30:* 442–450, 1966.

140. Svoboda, D., Grady, H., and Higginson, J. Aflatoxin B_1 Injury in Rat and Monkey Liver. Am. J. Pathol., *40:* 1023–1051, 1966.
141. Svoboda, D., Nielson, A., Werder, A., and Higginson, J. An Electron Microscopic Study of Viral Hepatitis in Mice. Am. J. Pathol., *41:* 205–224, 1962.
142. Svoboda, D. J., Racela, A., and Higginson, J. Variations in Ultrastructural Nuclear Changes in Hepatocarcinogenesis. Biochem. Pharmacol., *16:* 651–657, 1967.
143. Svoboda, D., and Soga, J. Early Effects of Pyrrolizidine Alkaloids on the Fine Structure of Rat Liver Cells. Am. J. Pathol., *48:* 347–373, 1966.
144. Swift, H. Cytochemical Studies on Nuclear Fine Structure. *In:* H. Busch (ed.), Experimental Cell Research, Suppl. 9, pp. 54–67. New York: Academic Press, 1963.
145. Thoenes, W., and Bannasch, P. Elektronen-und Lichtmikroskopische Untersuchungen am Cytoplasm der Leberzellen nach Akuter und Chronischer Thioacetamid-Vergiftung. Virchows Arch. Pathol. Anat. Physiol. Klin Med., *335:* 556–583, 1962.
146. Thomas, C. Zur Morphologie der Durch Diäthylnitrosamin Erzeugten Leberveranderungen und Tumoren bei der Ratte. Z. Krebsforsch., *64:* 224–233, 1961.
147. Timme, A. H., and Fowle, L. G. Effects of p-Dimethylaminoazobenzene on the Fine Structure of Rat Liver Cells. Nature, *200:* 694–695, 1963.
148. Turner, M., and Reid, E. An Anomalous Effect of Ethionine on Ribonucleic Acid Synthesis. Nature, *203:* 1174–1175, 1964.
149. Unuma, T., and Busch, H. Formation of Microspherules in Nucleoli of Tumor Cells Treated with High Doses of Actinomycin D. Cancer Res., *27:* 1232–1242, 1967.
150. Wattenberg, L. W. Chemoprophylaxis of Carcinogenesis: A Review. Cancer Res., *26:* 1520–1526, 1966.
151. Wogan, G. N. (ed.). Mycotoxins in Foodstuffs, pp. 153–273. Cambridge: Massachusetts Institute of Technology Press, 1965.
152. Wood, R. L. The Fine Structure of Hepatic Cells in Chronic Ethionine Poisoning and During Recovery. Am. J. Pathol., *46:* 307–330, 1965.

Figs. 1–24. All electron micrographs are from sections stained with lead.

Figs. 1–9. Acute.

Fig. 1. 3'-Methyldimethylaminoazobenzene, 24 hr. Both nucleoli show macrosegregation, with "caps". Note distinct and relatively pure separation of the fibrillar (*f*) and granular (*g*) components of the nucleolus. × 17,000.

Fig. 2. Tannic acid, 18 hr. The nucleolus at the right shows partial separation of the fibrillar (*f*) and granular (*g*) components but no "capping". The fibrillar component is situated within the nucleolus instead of at the periphery as in Fig. 1. In addition, numerous dense plaques are scattered throughout the nucleolar complex (*unmarked arrows*). The nucleolus at the left shows microsegregation. × 17,000.

Fig. 3. Dimethylnitrosamine, 24 hr. At this interval, the nucleolus is compact but partial separation of fibrillar and granular components (microsegregation) and dense plaques (*unmarked arrows*) are prominent. × 66,000.

Fig. 4. Dimethylnitrosamine, 48 hr. Microsegregation. The granular component of the nucleolus (*g*) is surrounded by stellate projections (*arrows*) consisting of mixed granules and fibrils with the latter predominating. × 55,000.

Fig. 5. Thioacentamide, 24 hr. Microsegregation. The distinction between the fibrillar (*f*) and granular (*g*) component can be made easily. The fibrils are condensed into several compact knots. × 55,000.

Fig. 6. Thioacetamide, 48 hr. Microsegregation. At this interval the nucleolus is compact and dense plaques are apparent within and at the periphery of the nucleolus (*unmarked arrows*). × 17,000.

Fig. 7. Aflatoxin, 24 hr. Numerous vesicles of smooth endoplasmic reticulum (*SER*) are apparent and ribosomes are not present. × 66,000.

Fig. 8. Dimethylnitrosamine, 24 hr. Three invaginations of the plasma membrane are apparent at the arrows. Each invagination has an indistinct coating along the margin. Interruptions of the plasma membrane are apparent (*asterisks*) and a single complete vesicle is present near the plasma membrane (*uppermost arrow*). × 21,000.

Fig. 9. Diethylnitrosamine, 24 hr. Several coated vesicles (*arrows*) are present in the cytoplasm distant from the plasma membrane. × 72,000.

Figs. 10–20. Chronic.

Fig. 10. Aflatoxin-induced tumor. The mitochrondria (*m*) are small and sparse with few matrix granules. Small aggregates of vesicles of smooth endoplasmic reticulum (*SER*) are apparent. Short and dilated segments of rough endoplasmic reticulum (*RER*) are scattered throughout the cytoplasm and occasional microbodies (*mb*) are apparent. Few lipid globules (*L*) are apparent. × 9600.

Fig. 11. Diethylnitrosamine-induced tumor. Extensive collections of vesicles of smooth endoplasmic reticulum (*SER*) are present as well as occasional cisterns of granular endoplasmic reticulum (*RER*). Multiple interruptions in the plasma membrane are noted at the unmarked arrows. × 7200.

Fig. 12. Diethylnitrosamine, 4 weeks after withdrawal. The most uniform abnormality is the presence of free ribosomes most abundant in the outlined areas. × 12,300.

Fig. 13. Dimethylnitrosamine, 6 weeks. The nucleolus is compact and shows knot-like condensations of the fibrillar component (*f*). × 12,000.

Fig. 14. Dimethylnitrosamine, 2 weeks. The mitochondria (*m*) are moderately swollen, and there is a reduction in the number of cristae. The orientation of the cristae (*arrows*) is often abnormal. × 14,000.

Fig. 15. Dimethylnitrosamine, 6 weeks. The mitochondrial abnormalities appear more severe with an increased degree of swelling, absence of matrix dense granules, and further reduction in the number of cristae. Close association of adjacent profiles is apparent. × 17,000.

Fig. 16. Dimethylnitrosamine, 6 weeks. Occasional mitochondria are extremely swollen with rupture of the limiting membranes (*asterisks*). A few remnants of cristae are apparent at the arrow. × 17,000.

Fig. 17. Dimethylnitrosamine, 4 weeks after withdrawal. In many cells nucleoli continue to show separation of the fibrillar and granular components. × 55,000.

Fig. 18. Ethionine 12 weeks. In the nucleoplasm there are condensations of interchromatin granules (*icg*), some forming thread-like structures. Perichromatin granules are evident in the square and at the arrows. The nucleolus in this section is not enlarged. The border of the nucleus is irregular. × 28,800.

Fig. 19. Ethionine-induced tumor. The mitochondria are smaller and fewer than normal with paucity of matrix dense granules. Several small microbodies (*mb*) are scattered throughout the cytoplasm. Most of the vesicles of endoplasmic reticulum have only few associated ribosomes. E, endothelial cell. × 17,000.

Fig. 20. Higher magnification of ethionine-induced tumor illustrates lipid globules (*L*) and only partially granulated membranes of endoplasmic reticulum (*arrows*). Elsewhere in the cytoplasm (*outlined area*), free ribosomes are apparent. × 7200.

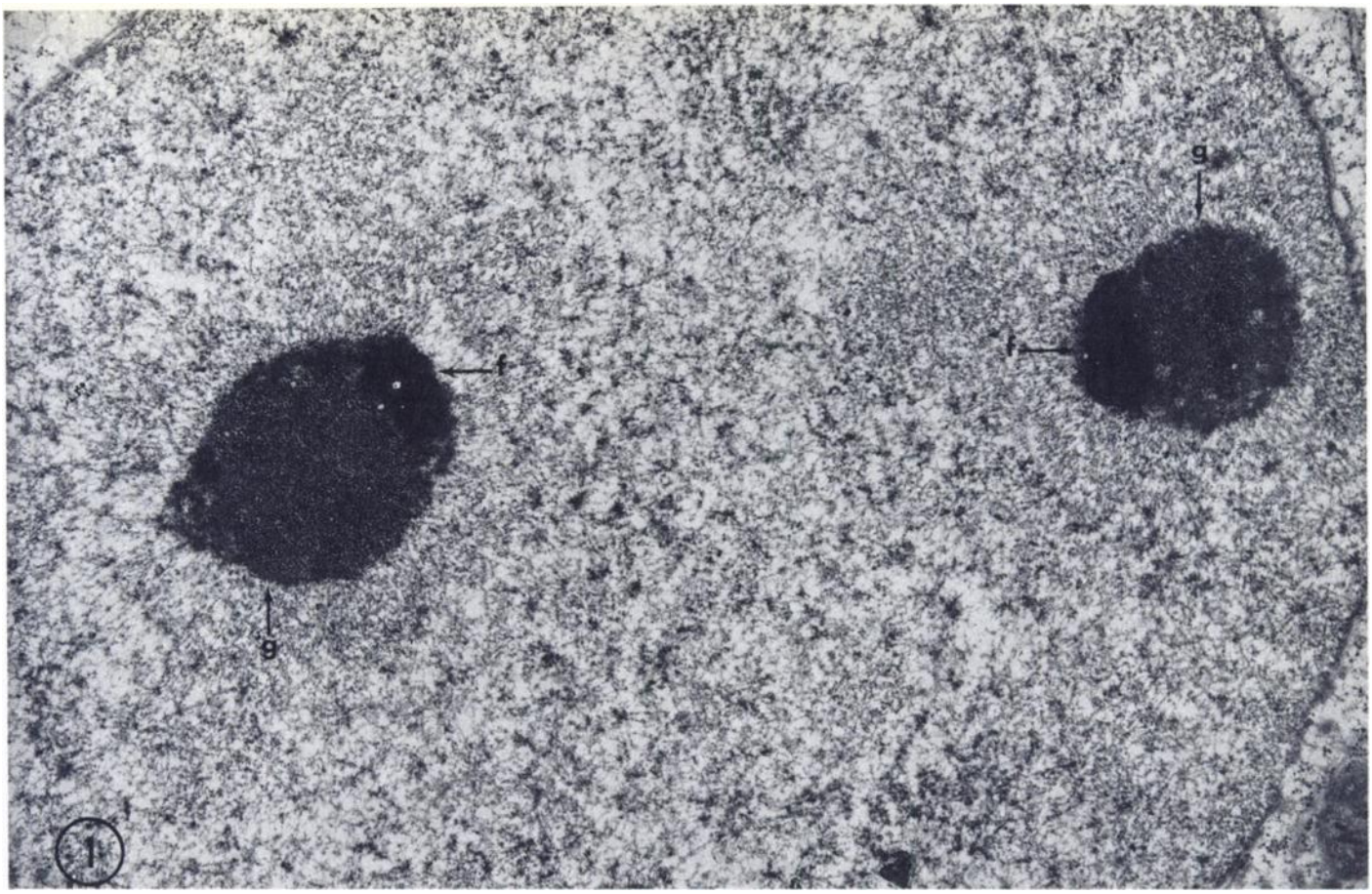
Figs. 21–24. Recovery.

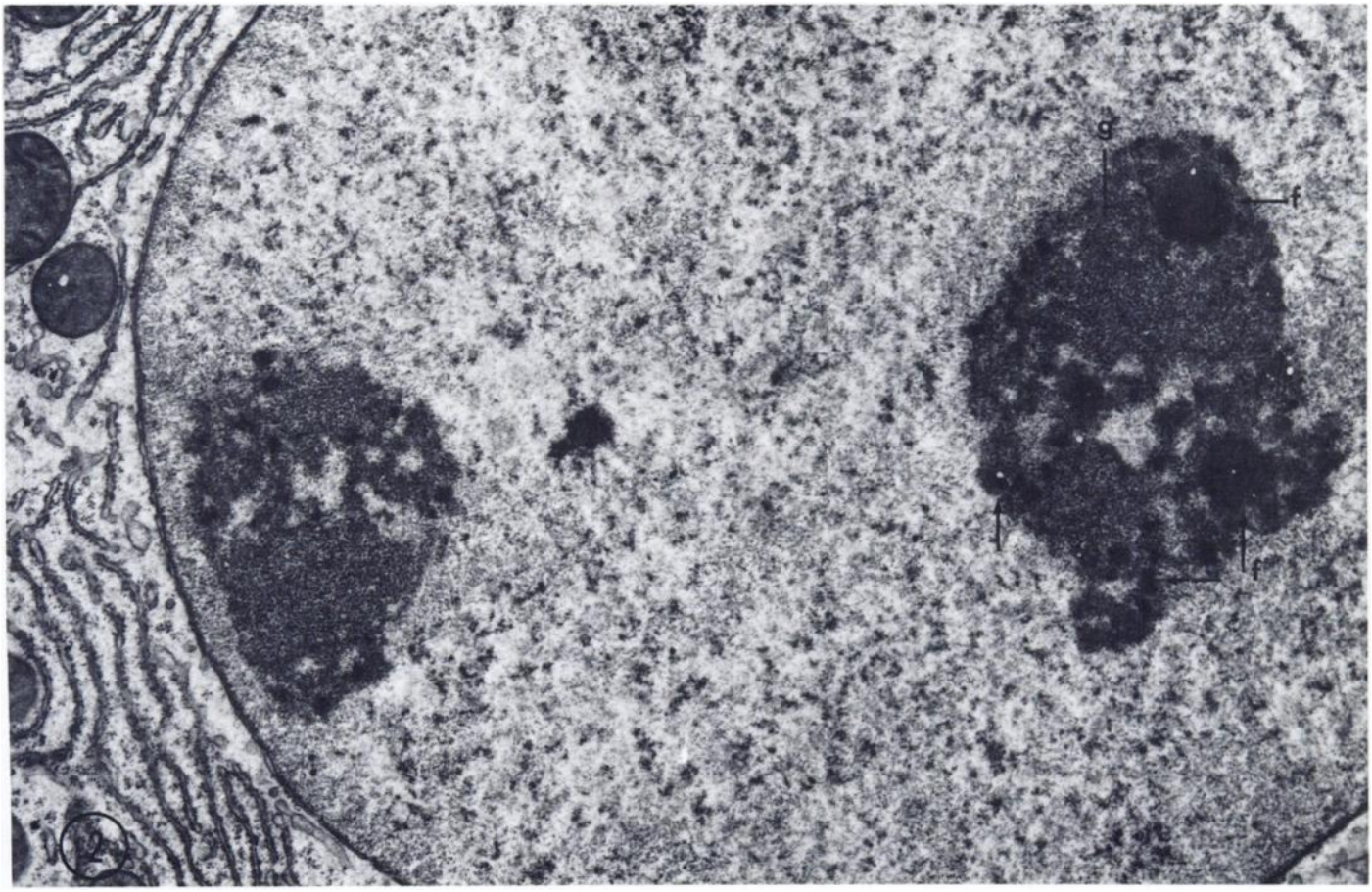
Fig. 21. Ethionine, 2 weeks after withdrawal. The mitochondrial profiles are irregular and numerous free ribosomes are apparent. × 17,000.

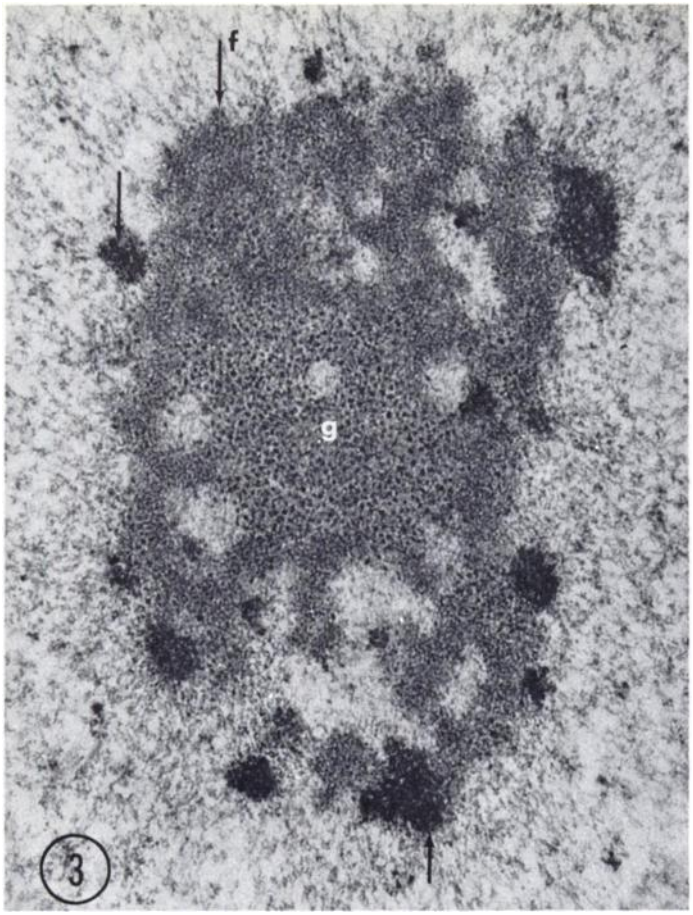
Fig. 22. Four weeks after withdrawal of ethionine. Although there is an increase in the number of vesicles of smooth endoplasmic reticulum, there is considerable recovery in the amount of granular endoplasmic reticulum. The mitochondria appear normal except for paucity in the number of matrix dense granules. × 18,500.

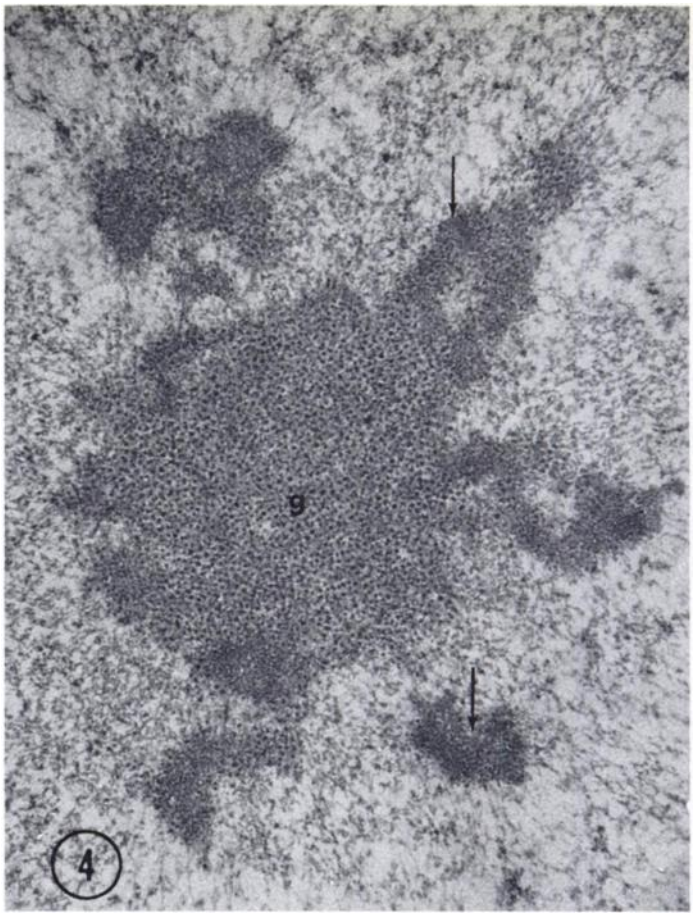
Fig. 23. 3'-Methyldimethylaminoazobenzene 4 weeks after withdrawal. The cells are essentially normal in their ultrastructure. × 7200.

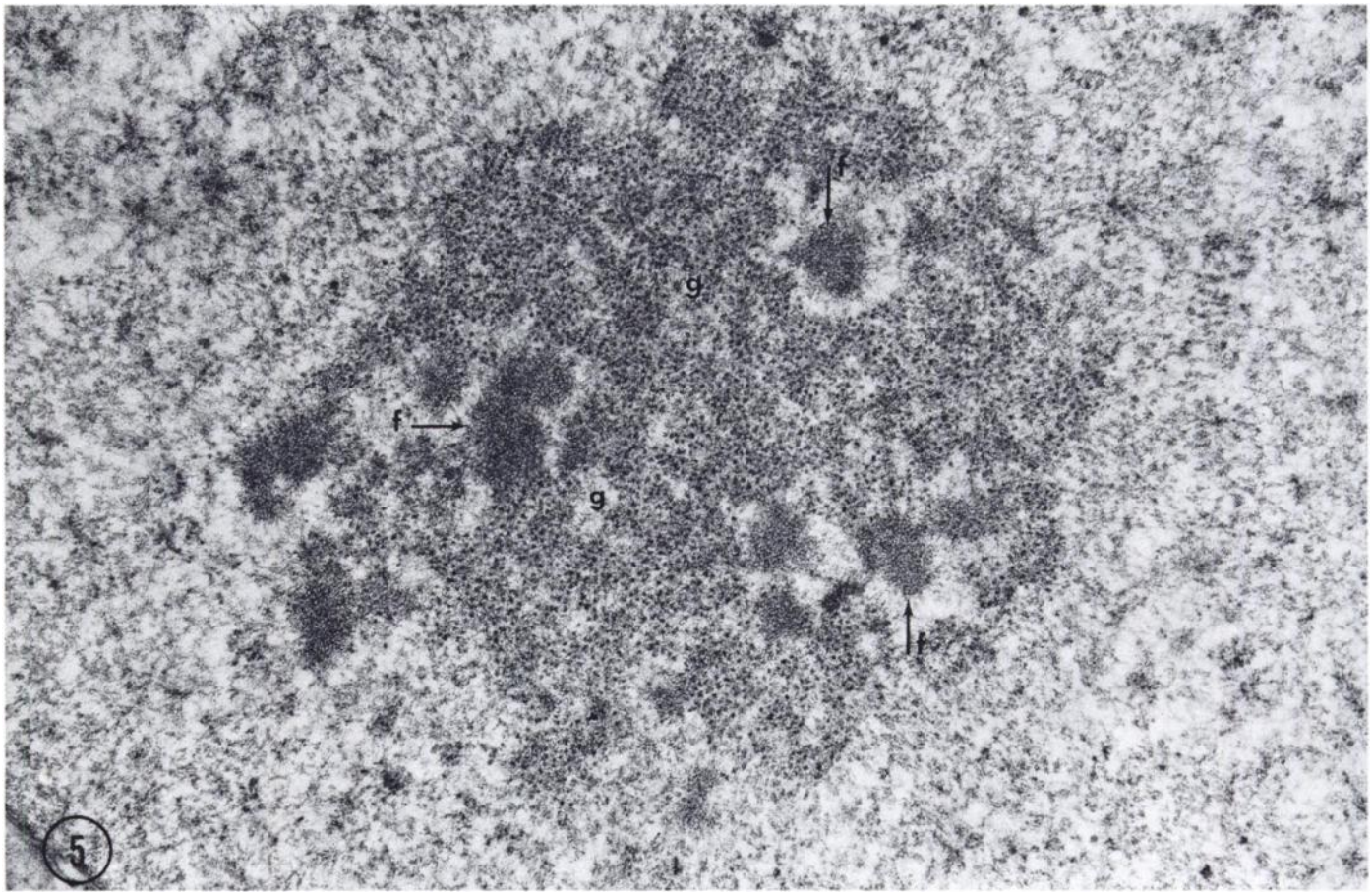
Fig. 24. Thioacetamide 6 weeks after withdrawal. The cells closely resemble the normal. × 7200.

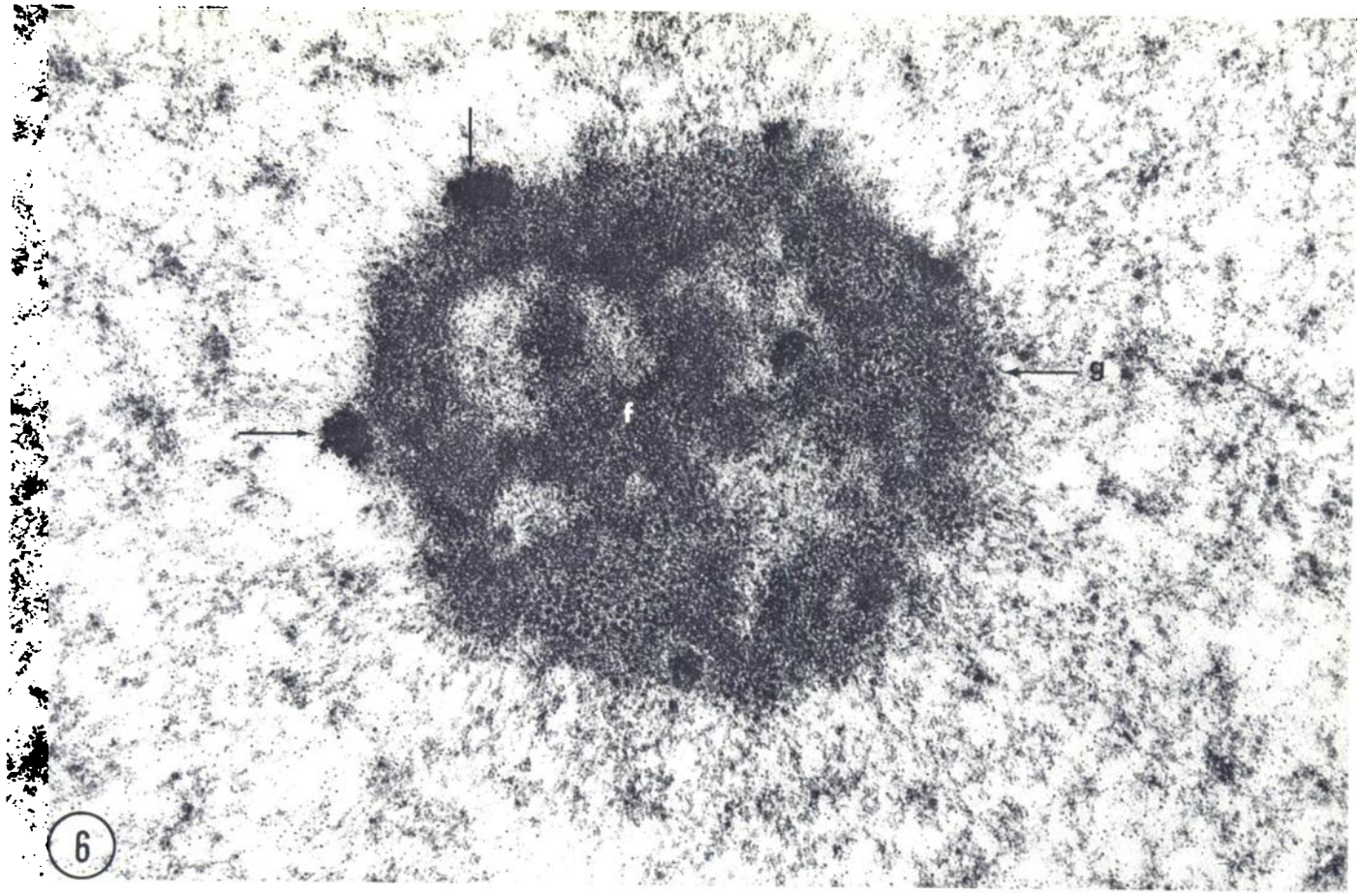


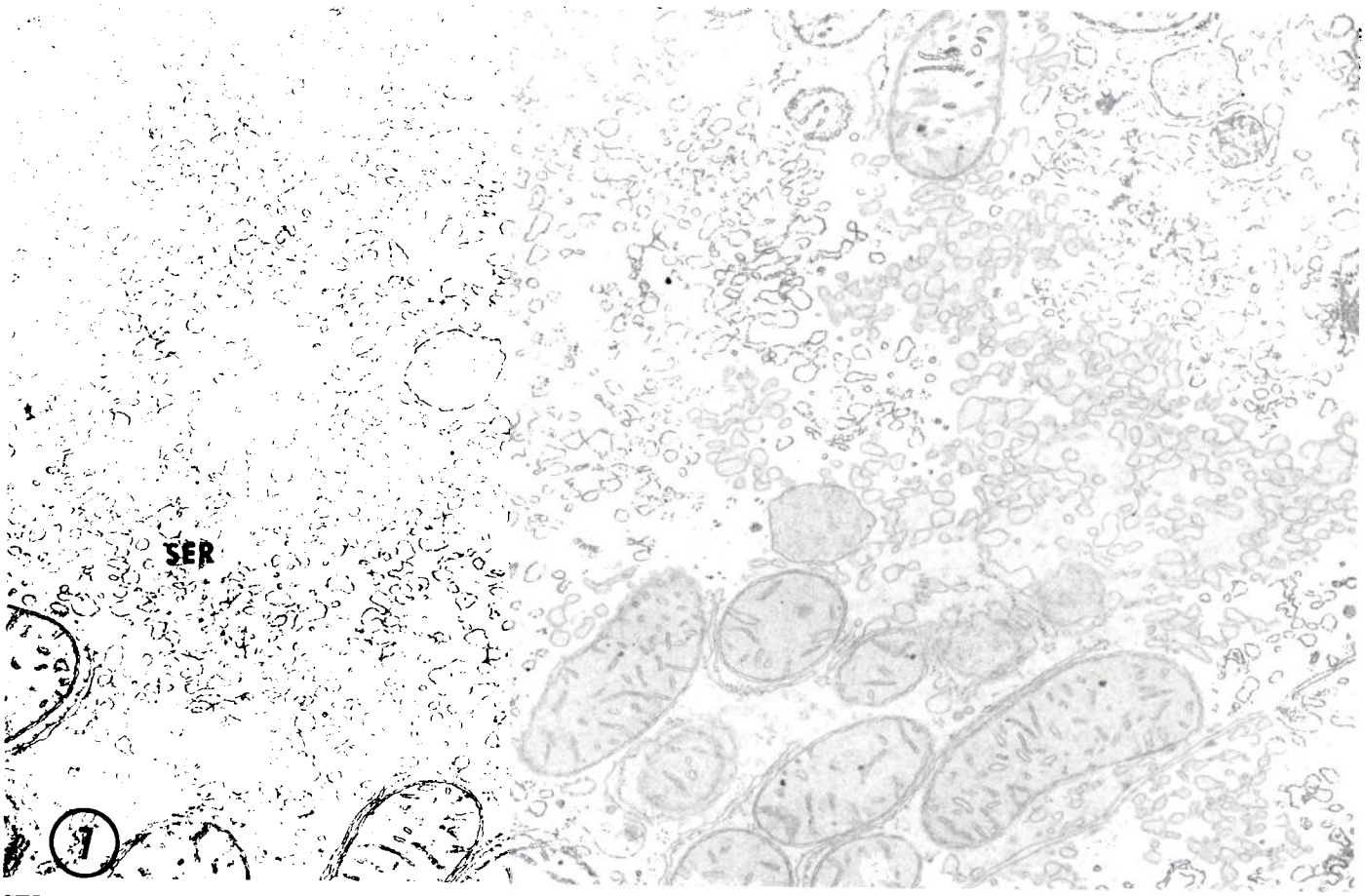


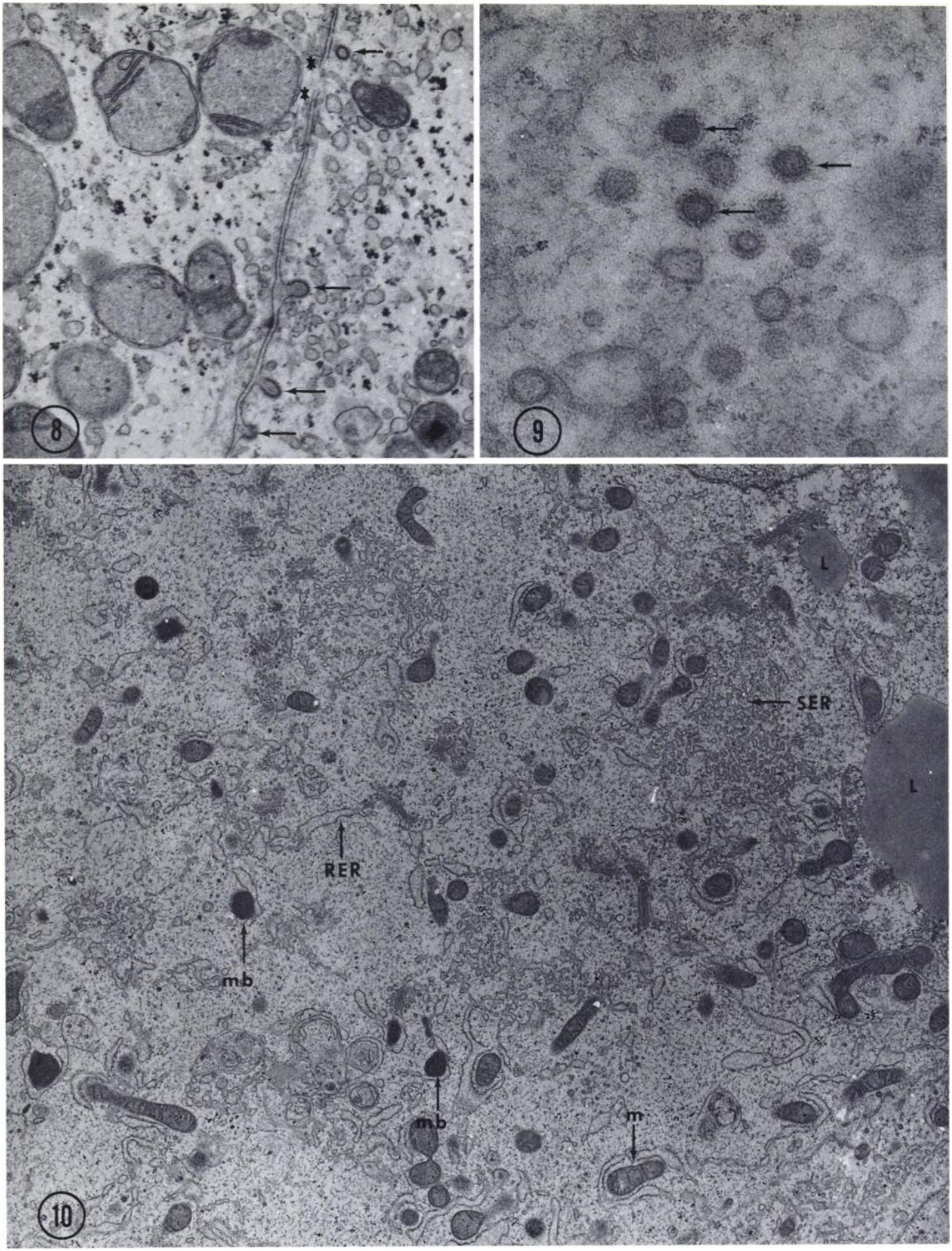


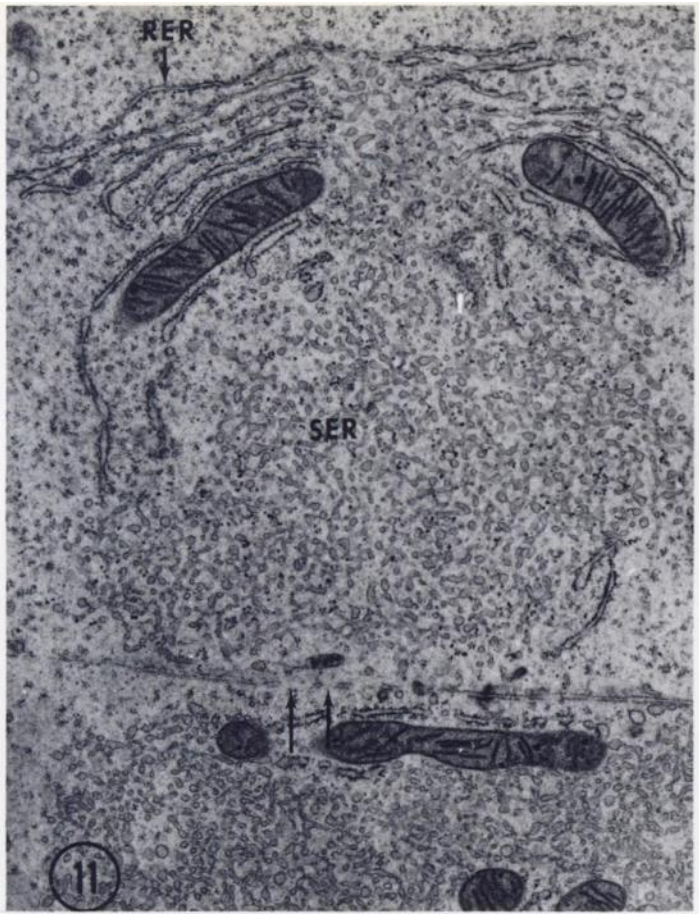


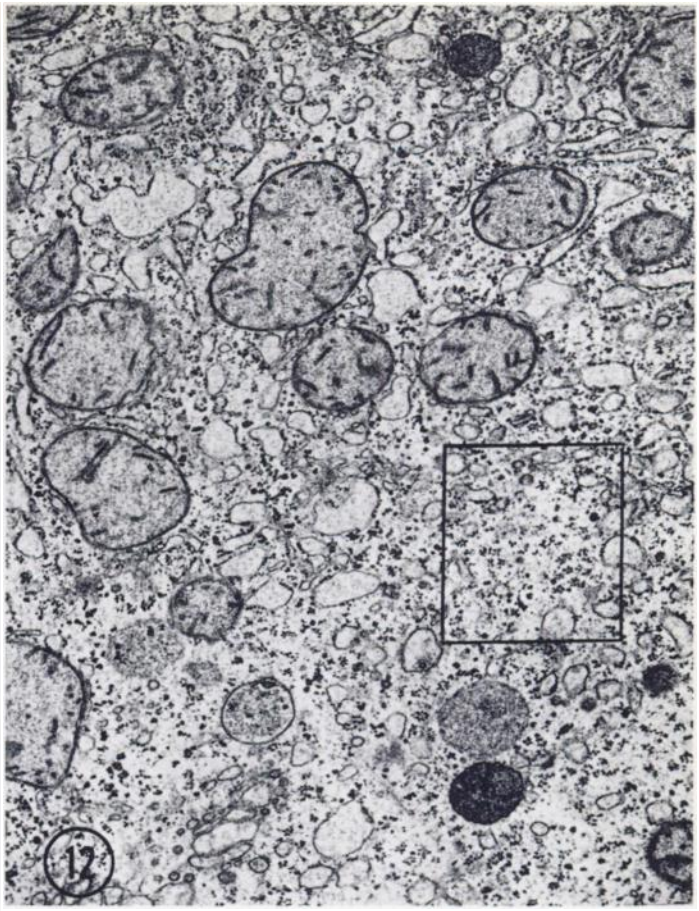


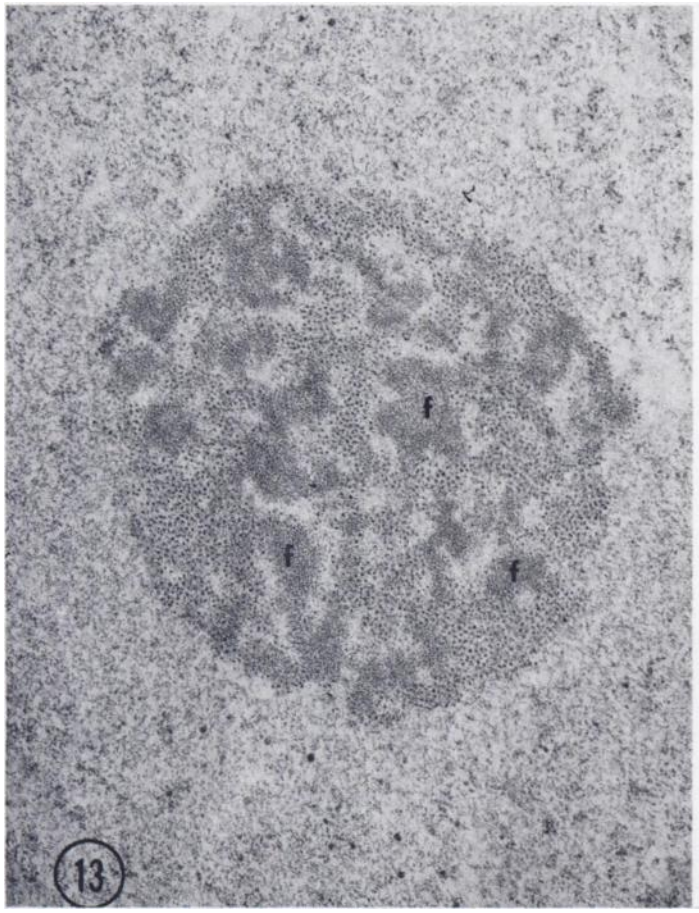


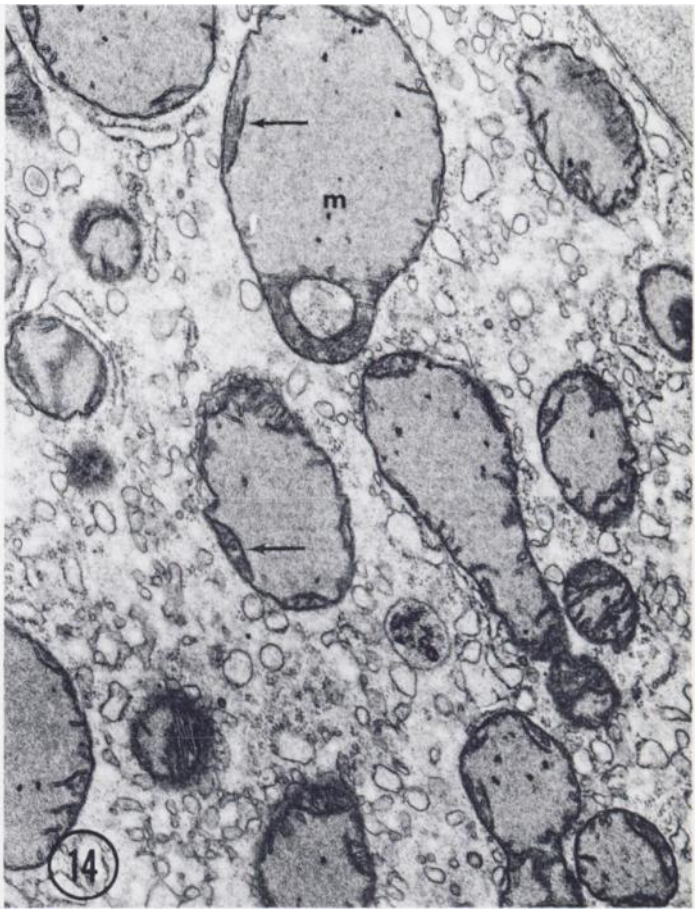


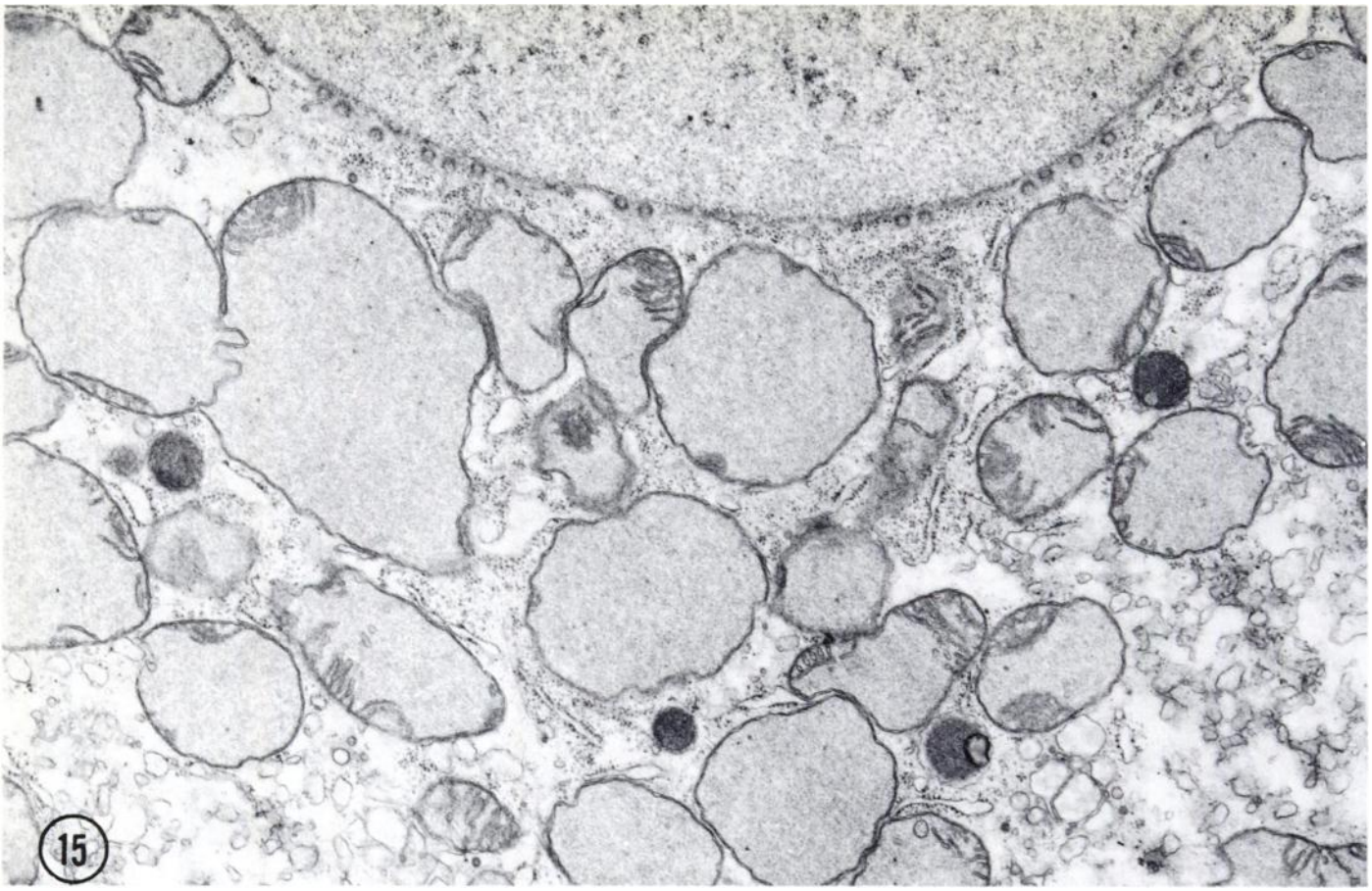




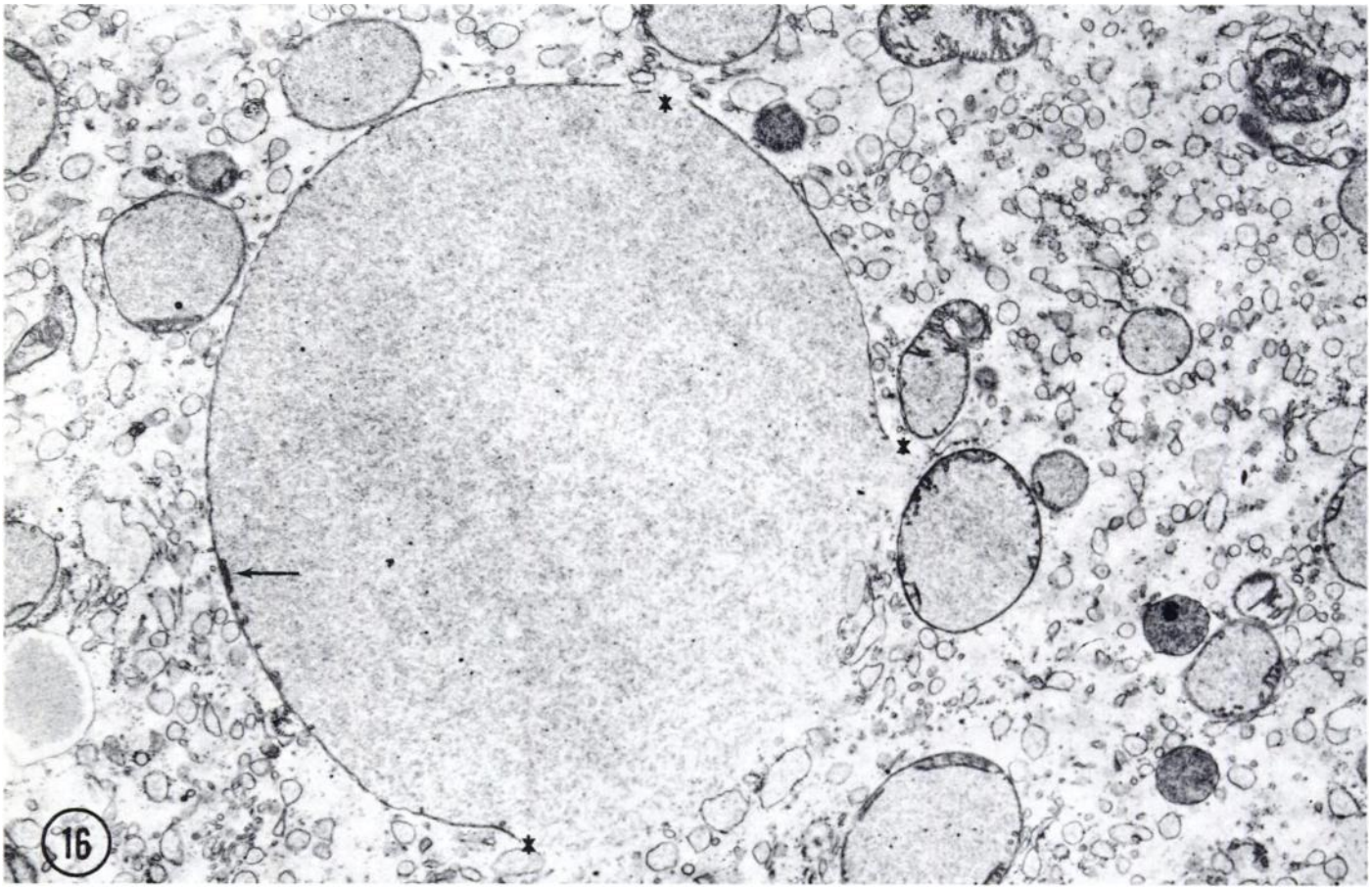




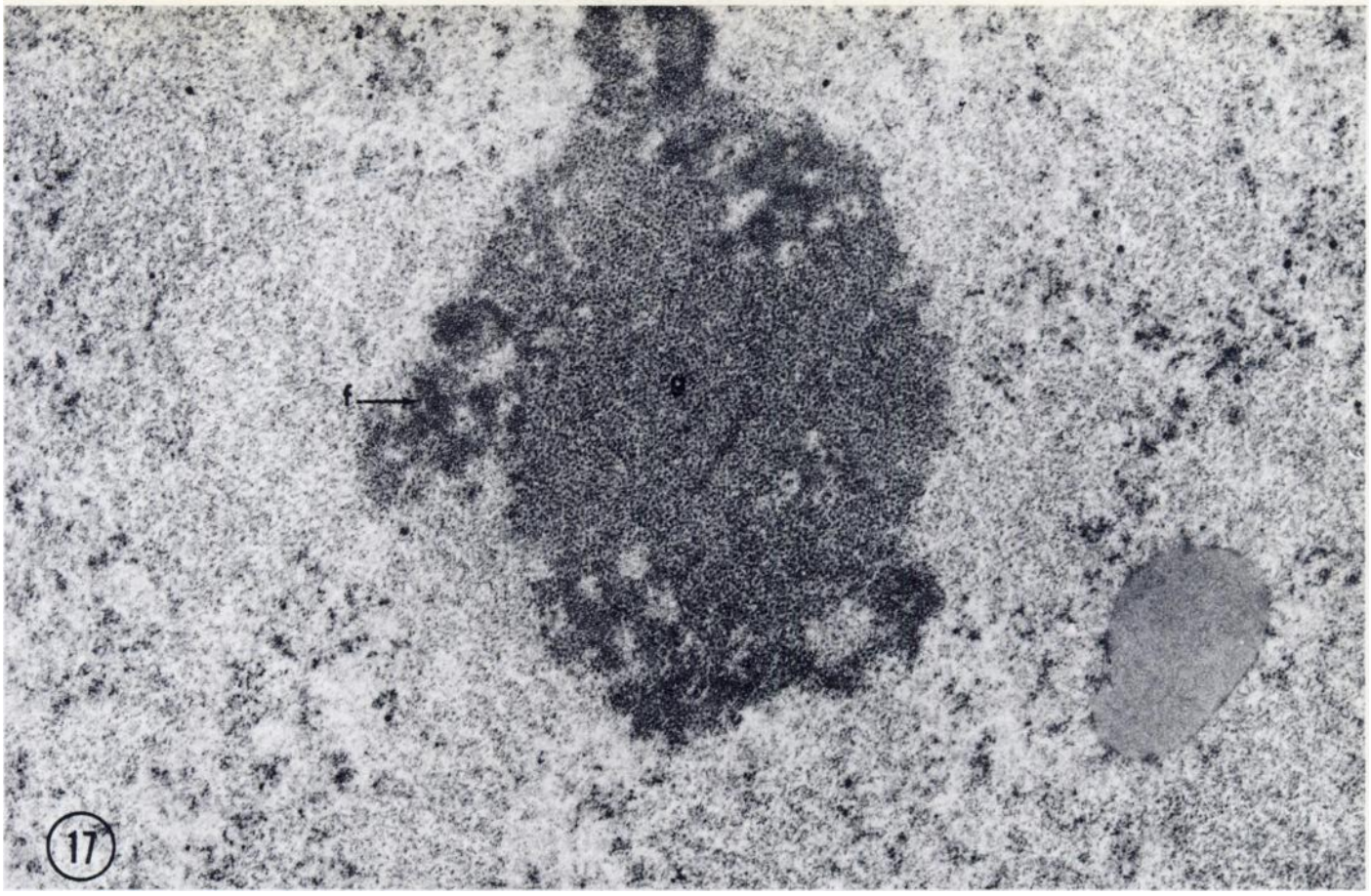




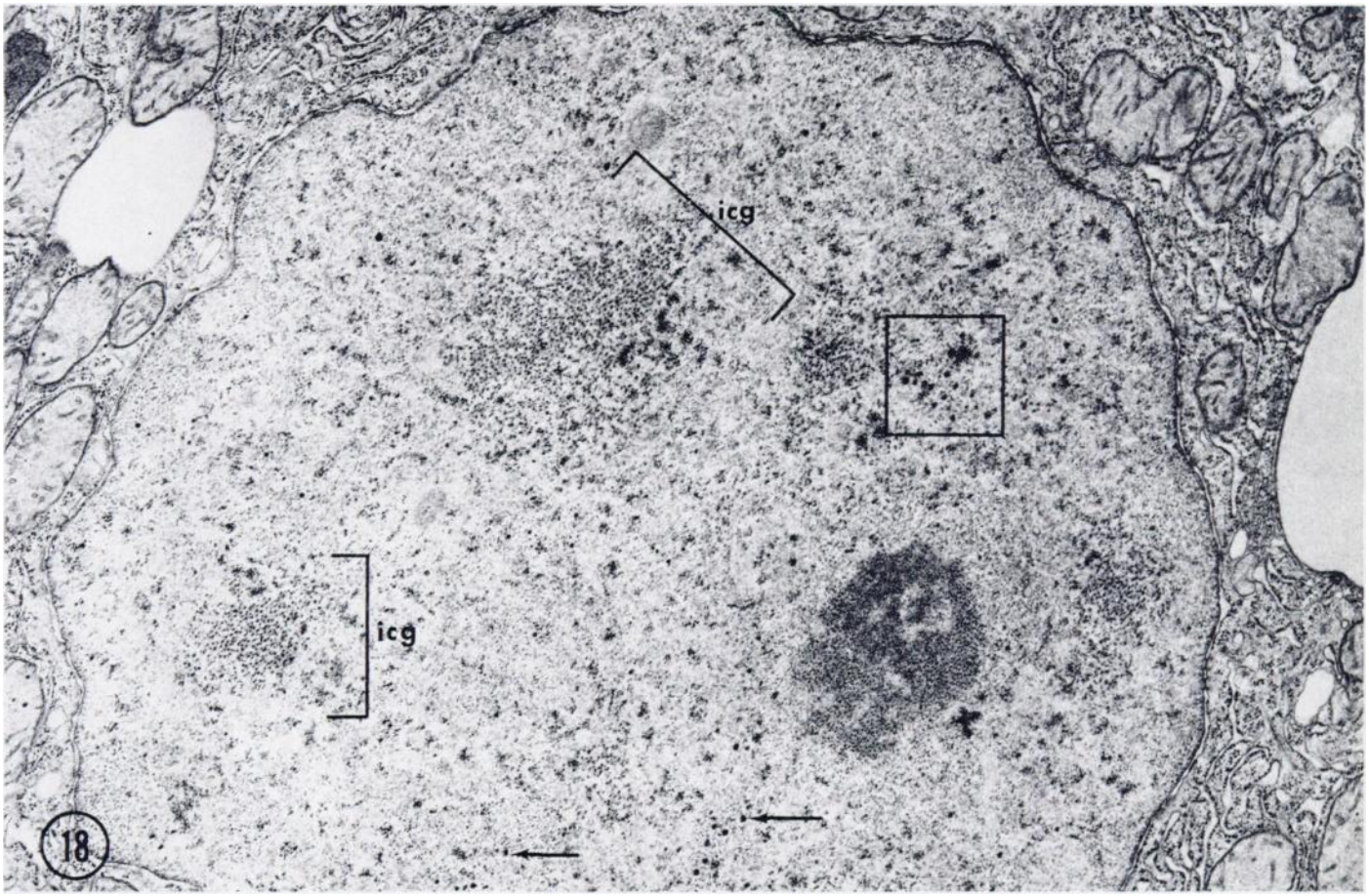
15



16



17



18