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VARIATIONS IN ULTRASTRUCTURAL NUCLEAR CHANGES IN HEPATOCARCINOGENESIS

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Abstract—The ultrastructural changes observed in the nuclei and nucleoli of liver cells following the acute and chronic administration of several hepato-carcinogens are discussed.

Three major types of nucleolar change have been observed following acute administration:

1. Macrosegregation—following aflatoxin, lasiocarpine and tannic acid.
2. Microsegregation—following DMN and 3′Me DAB.
3. Enlargement—following thioacetamide and ethionine.

In chronic toxicity nucleolar macrosegregation was not observed in precancerous liver or in tumours but some degree of microsegregation and nucleolar enlargement persisted.

The significance of these changes in the light of reported biochemical data on the action of these carcinogens is discussed, with special reference to DNA binding. It is concluded that neither the nucleolar nor the nuclear changes observed in both acute and chronic intoxication can be regarded as specific manifestations related to the carcinogenic process, in view of their apparent ability to regress and lack of consistency in chronic carcinogenesis.

PREVIOUS studies[1] have indicated that neoplasia is not related to a single mechanism, and it is generally accepted that multiple steps are involved, not only in initiating the carcinogenic process but also in promoting further growth of the neoplasm.[2] The wide variations in microscopic structure and biological behaviour of tumours produced by different carcinogens, even in tumours produced by the same carcinogen and arising in the same liver, are well known. While certain carcinogens of different chemical composition have produced relatively similar histological patterns,[3] there is some evidence in non-neoplastic liver that many of the histological changes may be epiphenomena, not necessarily related to hepatic neoplasia.[4, 5] Ultrastructural studies in hepatocarcinogenesis have been directed primarily to the study of cytoplasmic alterations,[6–15] and reports on the nuclear changes in acute and chronic experiments are limited.[16] The structural organization of the nucleus is less orderly and compartmented than that of the cytoplasm while its biological role is probably even more complex. Accordingly, despite improved and diversified methods for study of nuclear fine structure,[17] functional interpretations of structural changes in nuclei must be cautious and tentative. Nevertheless, as emphasized by Stowell,[18] attention must be given to the nucleus at the ultrastructural level before conclusions may be drawn regarding the significance of nuclear changes in carcinogenesis.

Many chemical carcinogens in acute toxic doses have produced morphological effects which have been regarded as being possibly specific for carcinogenesis.[19] In most studies, however, direct comparisons between the acute and chronic effects of carcinogens on nuclear ultrastructure have not been made.

This paper reports the results of experiments designed to compare the acute and chronic changes in nuclei in response to selected carcinogenic agents. The experiments were undertaken (i) preparatory to a series of studies to determine the effects of various carcinogens in combination, in order to identify those which would most likely cause synergistic responses as indicated by common ultrastructural reactions and (ii) to separate persistent from reversible changes, in view of the probability that the former would be more likely related to the carcinogenic process.

Emphasis is given to a comparison of the nuclear changes because of the primary role of the nucleus (and its constituents, DNA, RNA, histones, etc.) in initiating and regulating, via complex nucleo-ocytoplasmic exchange, such processes as protein synthesis, cell growth and division, phenomena of basic importance in neoplasia. The discussion is oriented to provide a base-line of the range of nuclear alterations in carcinogenesis and thereby place such alterations in a useful perspective for more detailed investigation.

The overall spectrum of changes observed will be reported in full elsewhere.[5]

MATERIALS AND METHODS

Inbred male F 344 rats were used in all experiments. The carcinogens used are given in Table 1a and 1b. They were administered at acute toxic and at chronic carcinogenic levels. Not all agents were studied in acute stages since their early effects have been reported previously by others.

TABLE 1A. SCHEDULE OF ACUTE EXPERIMENTS

Agent	Dose	Time of liver biopsy and sacrifice (hr)*
Aflatoxin B_1	0·45 mg/kg	24, 48, 72
Diethylnitrosamine (DEN)	140 mg/kg	24, 48, 72
Dimethylnitrosamine (DMN)	22·5 mg/kg	24, 48, 72
Lasiocarpine	80 mg/kg	1, 4, 6, 8, 12, 24, 48, 72
Tannic Acid	700 mg/kg	6, 18, 24, 48
Thioacetamide	60 mg/kg	18, 24, 48

* Two animals were studied at each interval.

Full details of the dose and duration of the experiments will be given elsewhere.[5] In the acute experiments, animals were examined at various intervals up to 72 hr. In the chronic experiments, the livers of animals were examined by laparotomy at fortnightly intervals until the time of death or until the appearance of tumours.

For light microscopy, tissues were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin, PAS and Feulgen's. For electron microscopy, samples of liver and tumours were fixed in cold 2% osmium tetroxide or glutaraldehyde buffered to pH 7·4 with *s*-collidine. After dehydration in a graded series of

alcohols, they were embedded in an epon-araldite mixture. Thin sections were cut using glass knives on an LKB ultramicrotome, stained with lead or uranium and examined in an RCA 3G electron microscope.

TALBE 1B. SCHEDULE OF CHRONIC EXPERIMENTS

	Agent	Dose	Duration (weeks)
(1)	Aflatoxin B_1	1 ppm in diet	33
(2)	Aflatoxin B_1	1 ppm in diet followed by 2 ppm	16 12
(3)	Diethylnitrosamine	0·55 mg/day	12, 23*
(4)	Dimethylnitrosamine	0·4 mg/day	24
(5)	Ethionine	0·25%	12, 24
(6)	3′ Methyl-Dimethylaminoazobenzene (3′—Me—DAB)	0·06%	14
(7)	Thioacetamide	0·032%	12, 24
(8)	Tannic Acid	40 mg/kg/day	18
(9)	Lasiocarpine	28 mg/kg/wk	9

* All animals sacrificed with tumours.

RESULTS AND DISCUSSIONS

At the doses used, the majority of agents, in acute experiments, produced a moderate degree of necrosis, the portion of the liver lobule involved depending on the particular carcinogen. In general, cytoplasmic alterations were minimal in chronic experiments. Aside from varying forms and severity of degenerative changes (fat accumulation, necrosis, focal cytoplasmic degradation and disorganization of the ergastoplasm), the most consistent cytoplasmic alteration at the ulstrastructural level, in both acute and chronic experiments with all agents, was detachment of ribosomes from membranes of endoplasmic reticulum and increase in the amount of smooth endoplasmic reticulum.

Acute and intermediate changes

Though some of the acute and sub-acute changes associated with high doses of carcinogens have been described,[6, 8, 11, 13–15, 20–27] this paper will present related observations in further detail.

For convenience, and without implying qualitative differences, the nuclear changes (Nucleus and Nucleolus) can be classified as follows:

1. *Macrosegregation* (nucleolar capping). In this category, nucleolar segregation occurred in large and rather distinct zones, some of which tended to form "caps" covering one or more peripheral aspects of the nucleolus. In almost all instances of this change, there was marked reduction in the diameter of the nucleolus. Typical macrosegregation was observed with aflatoxin and lasiocarpine (Figs. 1 and 2)*. With other agents, such as tannic acid, the clumps or aggregated dense zones tended to remain intranucleolar (Figs. 3 and 4), with the fibrillar zone becoming progressively smaller as the interval after administration became longer. With tannic acid, though light and dark zones occurred in the nucleolus, distinct compartmentation of granular

* All these and the following illustrations are from sections of rat liver stained with lead.

and fibrillar constituents into corresponding zones did not occur with the same degree of clarity as with aflatoxin, or lasiocarpine.

2. *Microsegregation.* In these situations, there was definite compact condensation of the fibrillar component of the nucleolus. These fibrillar condensations formed an interrupted or continuous peripheral rim about the granular interior (Figs. 5 and 6) or were multiple and plaque-like, situated amidst the granular constituents (Figs. 7 and 8). In contrast to macrosegregation which occurred in compact nucleoli, microsegregation occurred commonly, though not exclusively, in nucleoli that were dispersed. Microsegregation was observed with DMN (Figs. 6, 9 and 10) and 3′-Me-DAB (Fig. 5) in early stages. It was also apparent 1 hr after lasiocarpine (Figs. 7 and 8), prior to macrosegregation, and, in some cells, 18 hr after administration of tannic acid.

The essential difference between macro- and microsegregation lies in the size of the fibrillar areas and the degree of their separation from the granular component. In macrosegregation, the fibrillar component comprises a large, relatively pure and continuous zone adjacent to (Figs. 1 and 2) or within (Figs. 3 and 4) the granular area. In microsegregation, on the other hand, the fibrillar component appears as discontinuous, scattered and usually small condensations either at the periphery (Figs. 5 and 6) or amidst (Figs. 7–10) the granules.

3. *Nucleolar enlargement.* This change was typically seen with thioacetamide and ethionine. With thioacetamide (Fig. 11), there was marked enlargement of the nucleolus which tended to have a relatively homogeneous appearance although some distinction could be made between the granular and the fibrillar elements. The increase in the granular elements predominated over the fibrillar. With ethionine, both components appeared to increase in equal proportion (Fig. 12).

Chronic changes

Only the major morphological abnormalities will be described; normal structures are not discussed. In chronic experiments, macrosegregation was not seen with any of the carcinogens used, but microsegregation persisted in some liver cells of animals treated with DMN and 3′-Me-DAB while nucleolar enlargement was present with thioacetamide and ethionine.

In ethionine carcinogenesis, nucleolar enlargement was due apparently to an equal increase in both the fibrillar and granular components. There was also increase in interchromatin granules which often formed irregular, branching configurations (Fig. 13). Perichromatin granules also appeared to be increased in number but this was not confirmed quantitatively. By 10 weeks, nucleoli with membranes and cytoplasmic invaginations into the nuclei were seen occasionally. These changes persisted and were present in ethionine-induced tumor cells. The nuclear changes returned to normal in non-neoplastic portions of the liver within 6 weeks after withdrawal.

With DMN, variable degrees of nucleolar microsegregation were observed in non-tumor cells and, with DEN, the nuclei of tumour cells commonly contained dense granular collections, probably aggregates of interchromatin granules. The nucleolar changes in non-neoplastic liver reversed 4 weeks after withdrawal of the carcinogen. In 3′-Me-DAB-treated animals there was segregation with peripheralization of the fibrillar component surrounding the granular component (peripheral microsegregation) in some cells. In aflatoxin-treated animals, ultrastructural changes in the nucleus

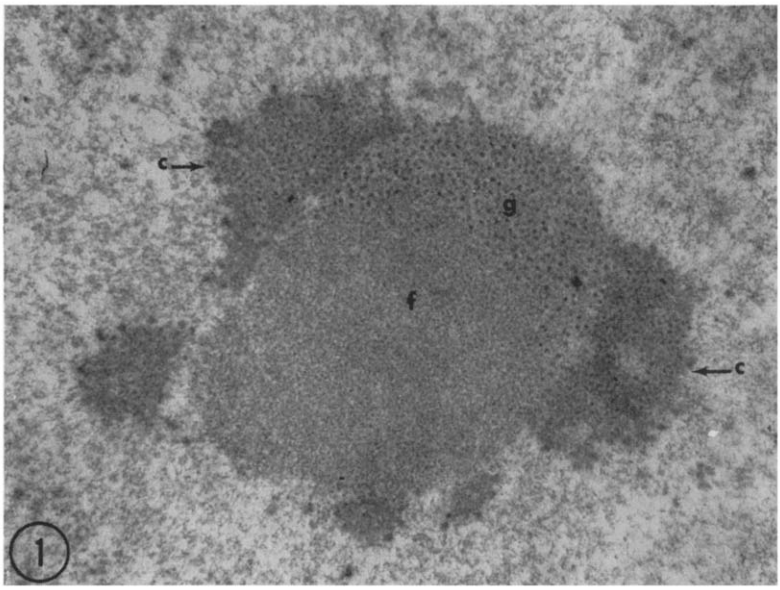

FIG. 1. Aflatoxin, 1 hr. A typical example of macrosegregation. The nucleolus is compact, smaller than normal and separated into distinct zones composed almost solely of fibrillar (f) or granular (g) elements. In addition, peripheral "caps" (c), composed of both fibrillar and granular constituents, are present. 41,500 ×.

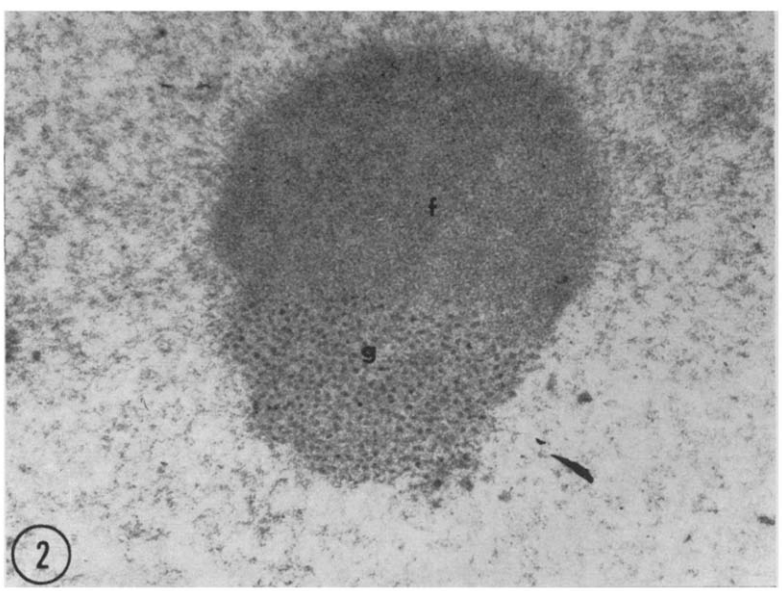

FIG. 2. Lasiocarpine, 12 hr. In this example of macrosegregation, peripheral nucleolar "caps" are not present but distinct demarcation into pure fibrillar (f) and granular (g) zones is evident. 41,500 ×.

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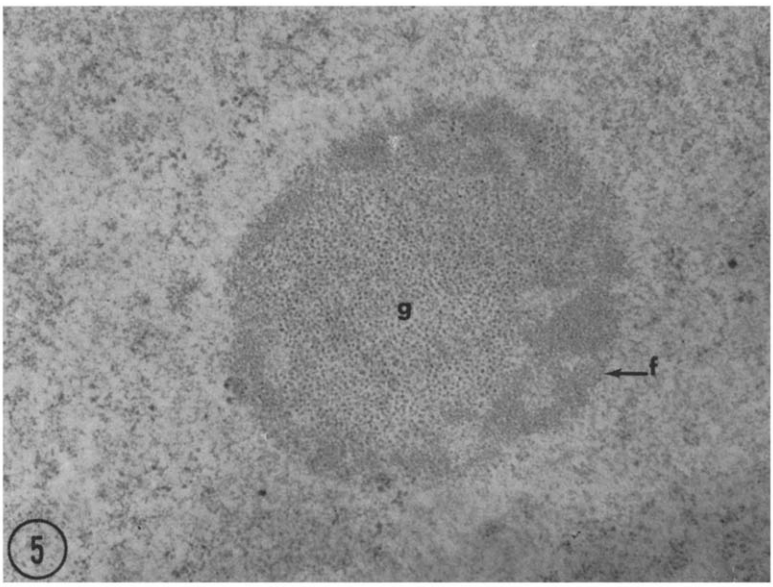

FIG. 3. Tannic acid, 18 hr. In this form of macrosegregation, the nucleolus is still compact and smaller than normal but the dense aggregates (d) remain in the interior of the nucleolus rather than at its periphery while the light areas (l) contain a mixture of both fibrils and granules and the distinct spatial separation of these two constituents is not so definite as in Figs. 1 and 2. 41,500 ×.

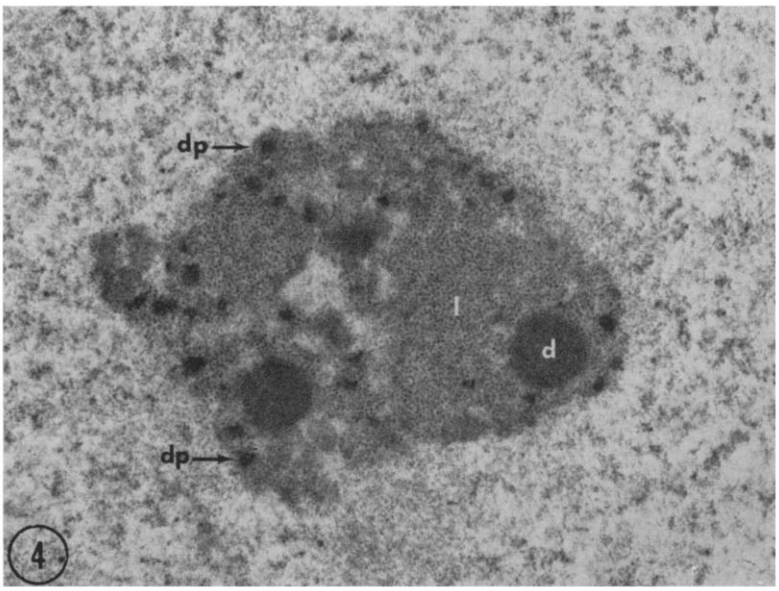

FIG. 4. Tannic acid, 18 hr. The dense areas (d) are in the main body of the nucleolus, as in Fig. 3. In addition, several dense plaques (dp) are apparent.

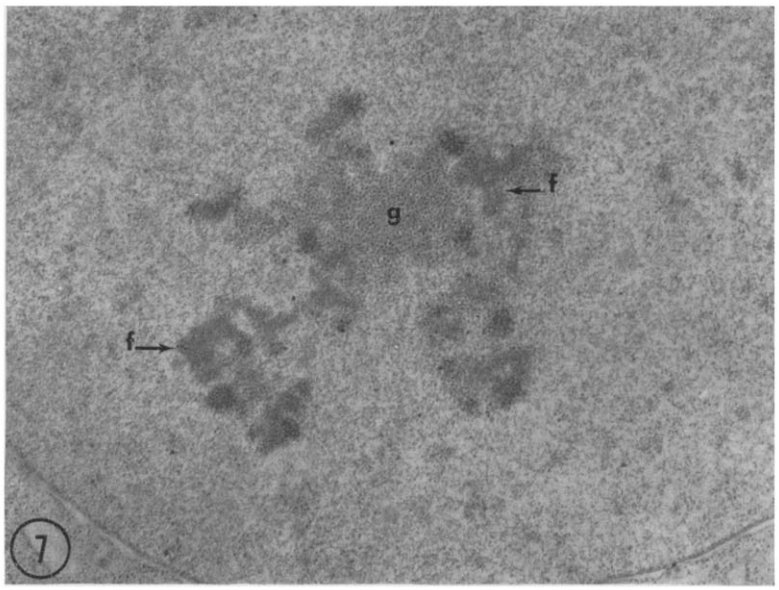

FIG. 5. 3′-Me-DAB, 2 weeks. Microsegregation in a compact nucleolus. The granular interior (g) is surrounded by an almost continuous narrow, diffuse margin composed predominantly of the fibrillar component (f). 21,000 ×.

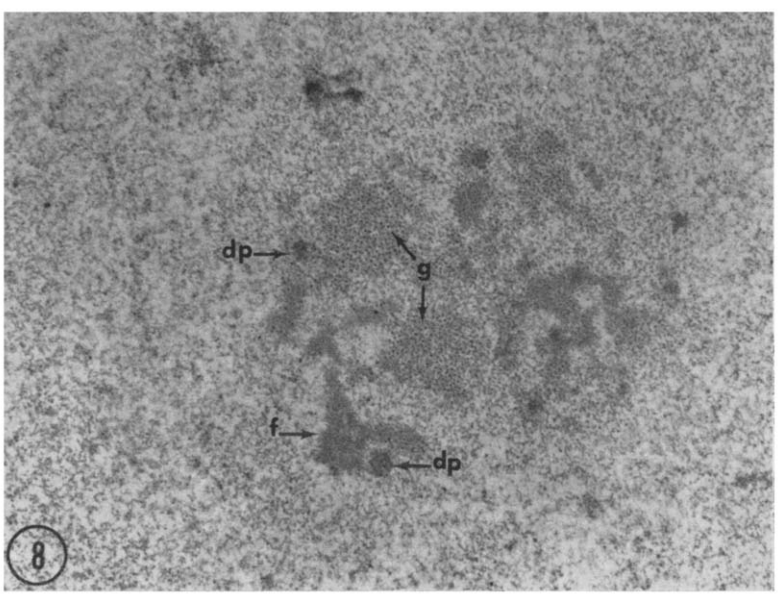

FIG. 6. DMN, 6 days. Microsegregation in a compact nucleolus. In this instance, the granular interior (g) is associated with focal, interrupted aggregates composed predominantly of the fibrillar (f) component. 21,000 ×.

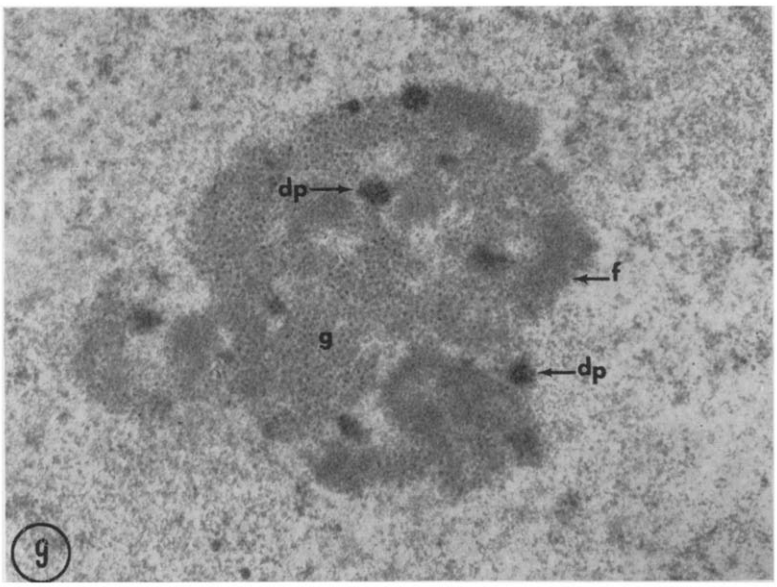

FIG. 7. Lasiocarpine, 1 hr. Microsegregation. The nucleolus, instead of being approximately circular and compact, is irregular and dispersed in serial ultra-thin sections. An irregular granular area (g) is associated with apparently random fibrillar (f) zones. The entire nucleolus is much smaller than normal. 10,700 ×.

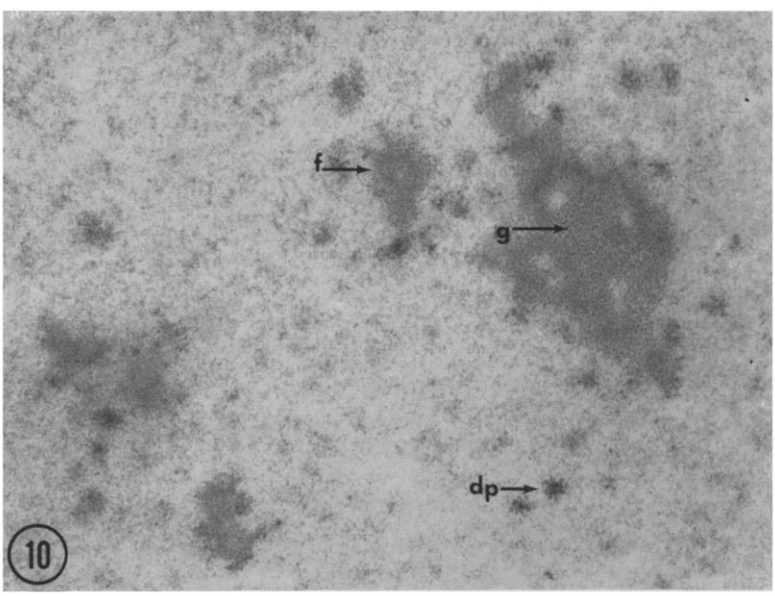

FIG. 8. Lasiocarpine, 1 hr. Microsegregation in a small, dispersed nucleolus. In addition to the granular (g) and fibrillar (f) areas as illustrated in Fig. 7, compact, circular satellite dense plaques (dp) are apparent. 10,700 ×.

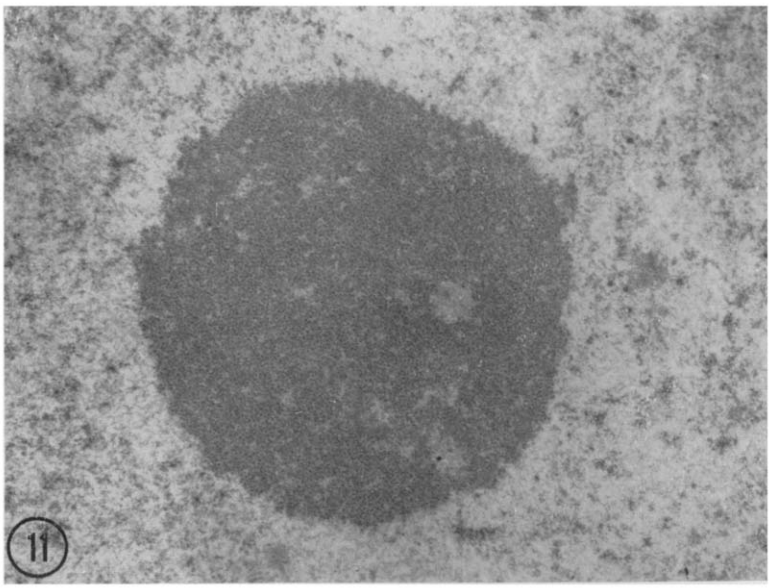

FIG. 9. DMN, 6 days. Microsegregation in a dispersed nucleolus. In addition to separation into granular (g) and fibrillar (f) areas several dense plaques (dp) are apparent in the interior and on the periphery of the nucleolus. 18,000 ×.

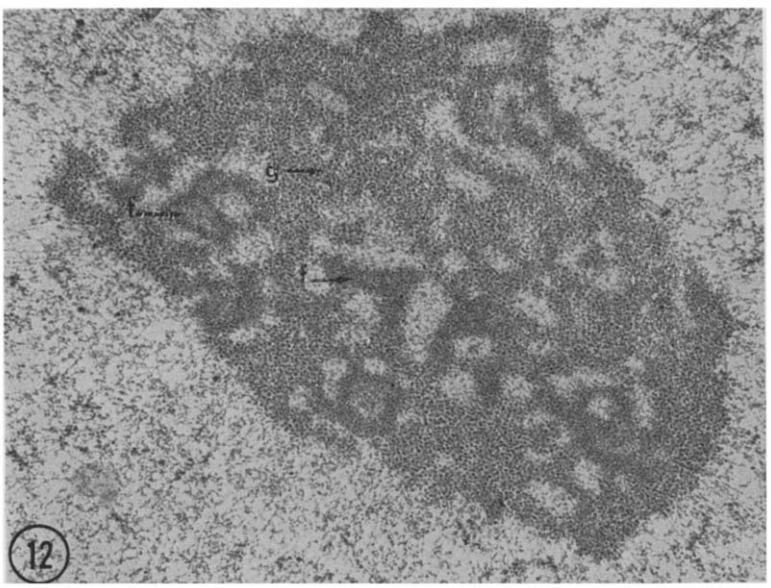

FIG. 10. DMN, 6 days. In this instance, microsegregation is apparent in a nucleolus smaller and more fragmented than in Fig. 9. Fibrillar (f) and granular (g) areas are apparent while dense plaques (dp) are widely separated from the parent nucleolus. In Figs. 8, 9 and 10, the dense plaques may represent condensations of the fibrillar areas. 41,500 ×.

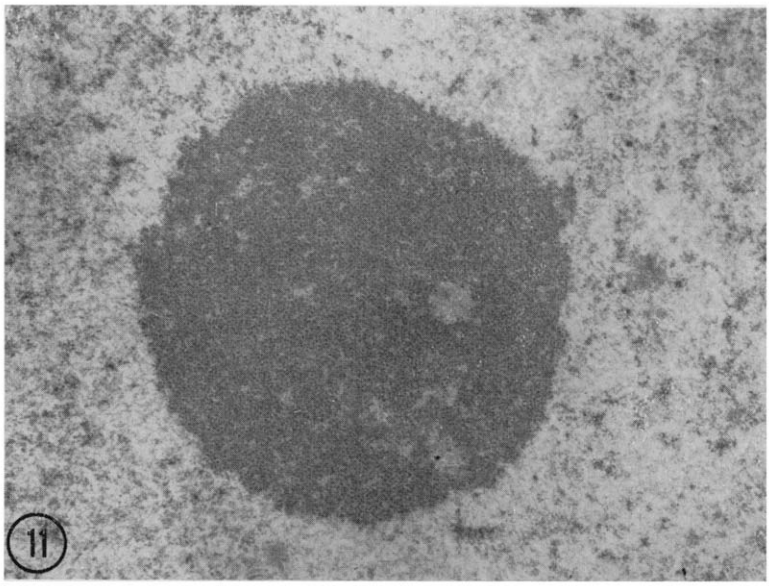

FIG. 11. Thioacetamide, 24 weeks. The nucleolus is enlarged due to a predominant increase in the granular constituent. 7,900 ×.

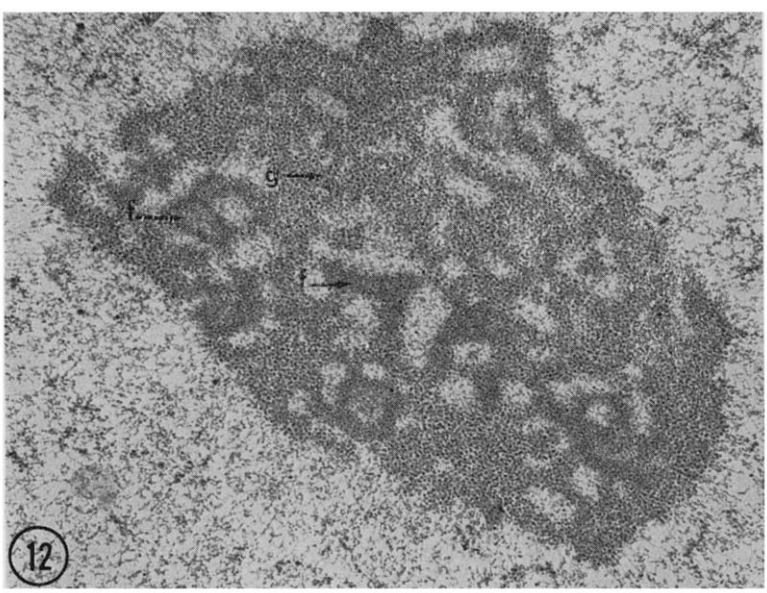

FIG. 12. Ethionine, 12 weeks. Nucleolar enlargement appears due to increase in both the fibrillar (f) and granular (g) constituents. 15,000 ×.

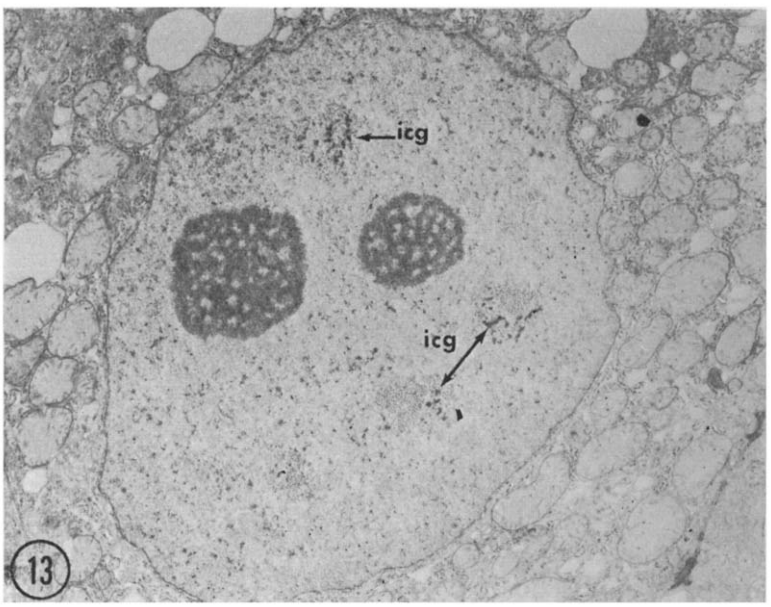

FIG. 13. Ethionine, 24 weeks. In addition to nucleolar enlargement, collections of increased interchromatin granules (icg) are apparent. 3600×.

were slight and macrosegregation, as observed in the acute experiments, was not seen. In non-neoplastic liver after removal of the carcinogen, no consistent change was present. In chronic experiments with tannic acid (18 weeks) and lasiocarpine (9 weeks) given in low but carcinogenic doses, the former appeared to cause a moderate increase in interchromatin granules, otherwise no consistent abnormalites in nuclear ultrastructure were apparent.

DISCUSSION

Nucleolar changes have been discussed in detail by Simard and Bernhard[26] who described decrease in size, segregation, and rearrangement of the nucleolar components into granular, fibrillar and amorphous zones and sometimes into a fourth highly-contrasted zone after administration of antimetabolites. These authors showed that macrosegregation was produced by antimetabolites which bind to DNA and interfere with DNA-mediated RNA synthesis. The present studies add further to the number of agents which produce some degree of nucleolar segregation in acute and intermediate stages.

Both DEN and DMN have been shown to alkylate nucleic acids while the mechanism of action of tannic acid has not been established. The alterations produced by 3′-Me-DAB resemble those illustrated by Simard and Bernhard in their Fig. 4. These authors[26] described a morphological sequence for the development of nucleolar segregation commencing with scattered aggregates of the fibrillar component which eventually united to form a fibrillar cap or zone, sometimes covered by a contrasted dark zone. The present results are insufficient to confirm or disprove this morphological sequence but it is interesting to note that when certain agents such as DMN and aflatoxin were given at doses which produced comparable degrees of necrosis, the nucleolar changes differed. In no case was conspicuous capping, typical of aflatoxin, observed with DMN and DEN. It would appear that Simard and Bernhard's comprehensive hypothesis offers considerable clarification and suggests that, at least in the acute stage, several of the carcinogens used in our experiments produce basically similar patterns that may be a consequence of DNA binding. With specific regard to aflatoxin, early but reversible inhibition of DNA-directed RNA synthesis has been demonstrated recently.[28]

Among the chemical actions of ethionine[29–31] and DAB[32–34] is their binding with DNA and RNA. Consequently, one would suspect that, if the DNA binding interferes with DNA-dependent RNA synthesis, these agents, too, should cause some degree of macrosegregation. Such changes were not observed in our chronic experiments and neither ethionine nor 3′-Me-DAB have been tested in a sufficient range of doses and intervals in acute experiments to permit a conclusion.

The acute changes due to several carcinogens vary in detail, but certain agents such as lasiocarpine, tannic acid, aflatoxin and, to some extent, DMN, cause basically similar forms of nucleolar alteration. In contrast, thioacetamide leads to marked nucleolar enlargement whereas 3′-Me-DAB causes peripheralization of the fibrillar component without apparent structural change in interchromatin granules. Ethionine causes nucleolar enlargement due to an increase in both the fibrillar and granular components. Thus the changes were not identical among all agents and, as is clearly indicated in the literature, macrosegregation has been produced by substances which

have not yet been shown to be carcinogenic. At present it would appear preferable, therefore, to regard macrosegregation as indicative of DNA-binding and interference with its template activity in certain cases, but not a universal response to all carcinogens nor specific for them. On the other hand, of course, it cannot be concluded that DNA binding of this or other types may not be an integral part of the carcinogenic process. The difficulty in assessing the ultimate importance of macrosegregation is further emphasized by the failure to detect it in the chronic stages of carcinogenesis and within tumours themselves. With aflatoxin, lasiocarpine, DMN and tannic acid this abnormality was not present in pre-cancerous or in tumorous liver. However, nucleolar enlargement due to thioacetamide and ethionine persisted throughout the experimental interval and, especially with ethionine, large nucleoli and increased interchromatin and perichromatin granules were conspicuous in tumors. Similarly, microsegregation due to 3′-Me-DAB and, to a lesser extent, DMN was present in some cells during intermediate and chronic stages. This lesion, however, has not been previously characterized nor studied sufficiently to comment on its possible significance.

In summary, no single uniform and persistent nucleolar alteration was present with any of the carcinogens studied. Thus, considering only chemical carcinogenesis in the liver, it is apparent that, with ultra-structural methods, no evidence for a common mechanism within the nucleus could be discerned and that nucleolar macrosegregation was not a feature related to carcinogenesis. Gelboin *et al.*[28] have alluded to the difficulty in relating carcinogenicity to interference with DNA-directed RNA synthesis and certain additional morphological examples serve to illustrate this point. With tannic acid, nucleolar macrosegregation in renal tubular epithelial cells is a prominent change[35] despite the fact that tannic acid does not typically cause renal tumors. In addition, anthramycin and lasiocarpine cause macrosegregation in reticulum cells of the spleen,[35] though neither agent is suspected as a carcinogen for the reticuloendothelial system.

Elsewhere, we have indicated that there are no consistent cytoplasmic changes with the exception of hyperplasia of smooth endoplasmic reticulum and ribosome detachment.[5] It should be noted that similar conclusions have been reached by others studying transplantable tumours where wide variations in morphology have been observed in different generations of a tumour.[36] The absence of any form of macrosegregation in the chronic experiments is not completely conclusive since it is possible that such changes may occur in only a few cells and will be missed, due to the limitations of sampling by electron microscopy. On the other hand, this nucleolar change is not seen within the tumours nor in hyperplastic nodules induced with any of the agents. In this context, it is of interest that, in malignant liver tumours induced with 3′-Me-DAB, preliminary experiments indicate that adequate doses of agents such as anthramycin[35] or lasiocarpine, which typically cause macrosegregation, do not evoke this change in the nucleoli of tumour cells. It would appear, therefore, that the capacity for segregation is lost in nucleoli of some malignant cells and the study of Tanaka *et al.*[37] indicates that nucleolar "caps" are not an inherited feature of cultured liver cells.

In conclusion, in view of the apparent ability of most of the acute nucleolar and nuclear lesions to regress and their lack of consistency in the chronic stages, it is preferable, at the present time, to regard them as non-specific in carcinogenesis.

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