Eur J Cancer Clin Oncol. Vol. 18. No. 3. pp. 291–306. 1982
Printed in Great Britain.

0277-5379/82/030291-15$03.00/0

Cytochemical and Autoradiographic Study of the Early Nuclear Lesions Induced by an Ellipticine Derivative in Isolated Rat Hepatocytes

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Abstract—*Isolated rat liver cells maintained in primary culture were treated with* 9-OH *ellipticinium* (9-OH E+). *The effects of this drug on the nuclear ultrastructure and on the chromatin transcriptional activity were studied by the combination of cytochemistry with high resolution autoradiography. At a low concentration* (1 μg/ml) *for periods ranging from* 10 min *to* 3 hr, 9-OH E+ *induced chromatin clumping, nucleolar microsegregation and a diminution in the number of perichromatin and interchromatin fibrils. Autoradiography revealed that this compound inhibited rapidly the incorporation of* [5-^{3}H]-*uridine in the nucleus, preferentially but not exclusively in the nucleolar area. In addition, the distribution of the radioactivity in the nucleoli proved that the processing of the pre-ribosomal ribonucleic acids (pre-rRNA) synthesized in the presence of the drug was blocked while the processing during* 9-OH E+ *treatment of normally synthesized pre-rRNA was not altered. These findings suggest that the inhibition of pre-rRNA processing might result from an impairment of factors controlling this processing rather than from a direct action of* 9-OH E+ *on pre-rRNA molecules.*

INTRODUCTION

ELLIPTICINE (E) is a natural substance which has been extracted from plants belonging to the botanical family of Apocynaceae, such as *Ochrosia elliptica.* The dimethyl pyridocarbazole structure of this alkaloid endows it with intercalative properties towards DNA [1, 2]. The antitumoral activity of E [3], initially mentioned by Dalton *et al.* [4] and by Hartwell *et al.* [5], has been subsequently studied by LeMen *et al.* [6], Mathé *et al.* [7] and Le Pecq *et al.* [8]. The chemotherapeutic index appeared sufficiently interesting to initiate clinical trials, but these were soon interrupted because of undesirable secondary effects such as cardiovascular lesions or cerebellar ataxia.

Ellipticiniums (E+) are synthetic substituted derivatives bearing a quaternarized ammonium [9]. The affinity of these molecules to DNA is much (up to 100-fold) higher than that of the natural alkaloid. At the same time, the antitumoral activity is increased [10, 11], which has stimulated renewed interest for the continuation of clinical trials. However, only a few works deal with the morphological effects of these drugs on cell structure. Dufer *et al.* [12] studied by means of optic microscopy the modifications of leukocytes in rats after 10 days of treatment with methoxy-9-ellipticine. They histochemically detected quantitative variations in acid phosphatases and esterases. Structural abnormalities of mitochondria were reported at the electron microscope level in HeLa cells treated with E derivatives [13], as well as in L 1210 cells [11]. Concerning the nucleus, multilobation [11] and appearance of punctiform chromatin [13] were observed by the same authors. But, to our knowledge, no precise ultrastructural description has been made of nuclear lesions.

Isolated rat liver cells maintained *in vitro* offer a suitable experimental model for studying modifications of nuclear structural components. Their nuclear morphology remains

Accepted 20 October 1981.
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for at least 36 hr similar to that of *in situ* hepatocytes, with clearly defined nucleolar, condensed chromatin and interchromatin areas. Such structural features facilitate the observation of ribonucleoproteic (RNP) structures after appropriate staining. In addition, these cells lend themselves easily to labeling. Consequently, several studies carried out recently on isolated rat liver cells resulted in a better knowledge of the functional significance of the different nuclear RNP structures [14].

The purpose of this experiment was to investigate, by means of the combination of cytochemistry with high resolution autoradiography, the early effects of E on the nucleus of isolated rat liver cells. Our findings are discussed in the light of recent results on metabolic and structural lesions induced by various inhibitors of RNA metabolism.

MATERIALS AND METHODS

Isolation of adult rat hepatocytes

Three-month-old male rats (strain WAG) were used after a fasting period of 18 hr. The hepatocytes were isolated as previously described [15]. The pellets of liver cells were then resuspended in Eagle's Minimum Essential Medium (MEM), supplemented with 10% calf serum (700,000 cells per ml). Three ml of this suspension were put into 25 cm^2 Falcon flasks. The incubation was carried out at 37°C for 3 hr in an atmosphere of 95% air and 5% CO_2. Then the medium was changed and the incubation carried out for about 20 hr before the beginning of the experiment.

Ellipticine treatment

The following compounds were used: ellipticine (E), 9-OH ellipticine (9-OH E) and the same substances with a quaternarized ammonium, i.e., ellipticinium iodo-methylate (E+) and 9-OH ellipticinium aceto-methylate (9-OH E+).

Twenty-five μl of a 2 mg/ml solution (either in water or in 10^{-2} M HCl or in 10% dimethyl sulfoxide) were added to 5 ml of medium in order to obtain a final concentration of 10 μg/ml. Concentrations of 5 μg/ml, 1 μg/ml and 0.1 μg/ml were prepared by 1/2 and successive 1/10 dilutions of the 2 mg/ml solution with water and addition of 25 μl of these solutions per flask. Controls were made by addition of 25 μl of each solvent used per flask.

Cells were treated at various concentrations from 5 min to 24 hr.

Electron microscopy

At the end of each experiment, the cells were fixed in the flask by replacing the medium with 1.6% glutaraldehyde in Sörensen's phosphate buffer (0.1 M, pH 7.4) at +4°C for 45 min.

The cells were then rinsed in the same buffer, scraped and sedimented by centrifugation (10 min at 3500*g*). Dehydration of the pellets was carried out in ascending concentrations of ethanol and Epon inclusion, achieved by the usual technique.

Thin sections were stained either with the EDTA regressive stain for ribonucleoproteins [3] or with uranyl acetate and lead citrate.

Autoradiography

Two types of experiments were carried out. In the first type, cells were incubated for periods of 15 min or 1 hr in ellipticine-containing medium and then labeled by addition of 80 μCi of triated uridine ([5-^{3}H]-uridine, C.E.A., Saclay, France; specific activity: 20–30 Ci/mmol). After 30- or 60-min exposures to [5-^{3}H]-uridine, the hepatocytes were briefly rinsed with serum-free MEM containing 1 mg/ml of non-radioactive uridine at 0°C. They were then fixed in 1.6% glutaraldehyde and embedded in Epon. Controls were treated in the same way in a medium deprived of ellipticine.

In the second type of experiment, the cells were labeled for 15 min in the presence of 100 μCi/ml of [5-^{3}H]-uridine, then rinsed briefly as above and post-incubated for 1 or 3 hr in the presence of ellipticine and 100 μg/ml of non-radioactive uridine. Controls were fixed immediately after labeling and following 1 or 3 hr of post-incubation in the presence of unlabeled uridine. Ilford L_4 emulsion was applied onto ultra-thin sections mounted on formvar-coated grids by the loop technique [16]. After 3–4 months of exposure, the autoradiographs were developed with the gold latensification technique [17]. The grids were then stained preferentially for RNP by the regressive stain of Bernhard [3] modified for autoradiography [18], or conventionally by uranyl acetate and lead citrate. A quantitative analysis of the incorporation of [5-^{3}H]-uridine in the nucleolar and extra-nucleolar areas and in the cytoplasm was carried out in the first type of experiment, following 30 min of labeling. Grain counting was effected on nuclei photographed under controlled magnification. Measurements of areas, determination of grain densities and statistical calculations and comparisons were performed by using a Hewlett–Packard 9820 calculator fitted to a graphic digitizer.

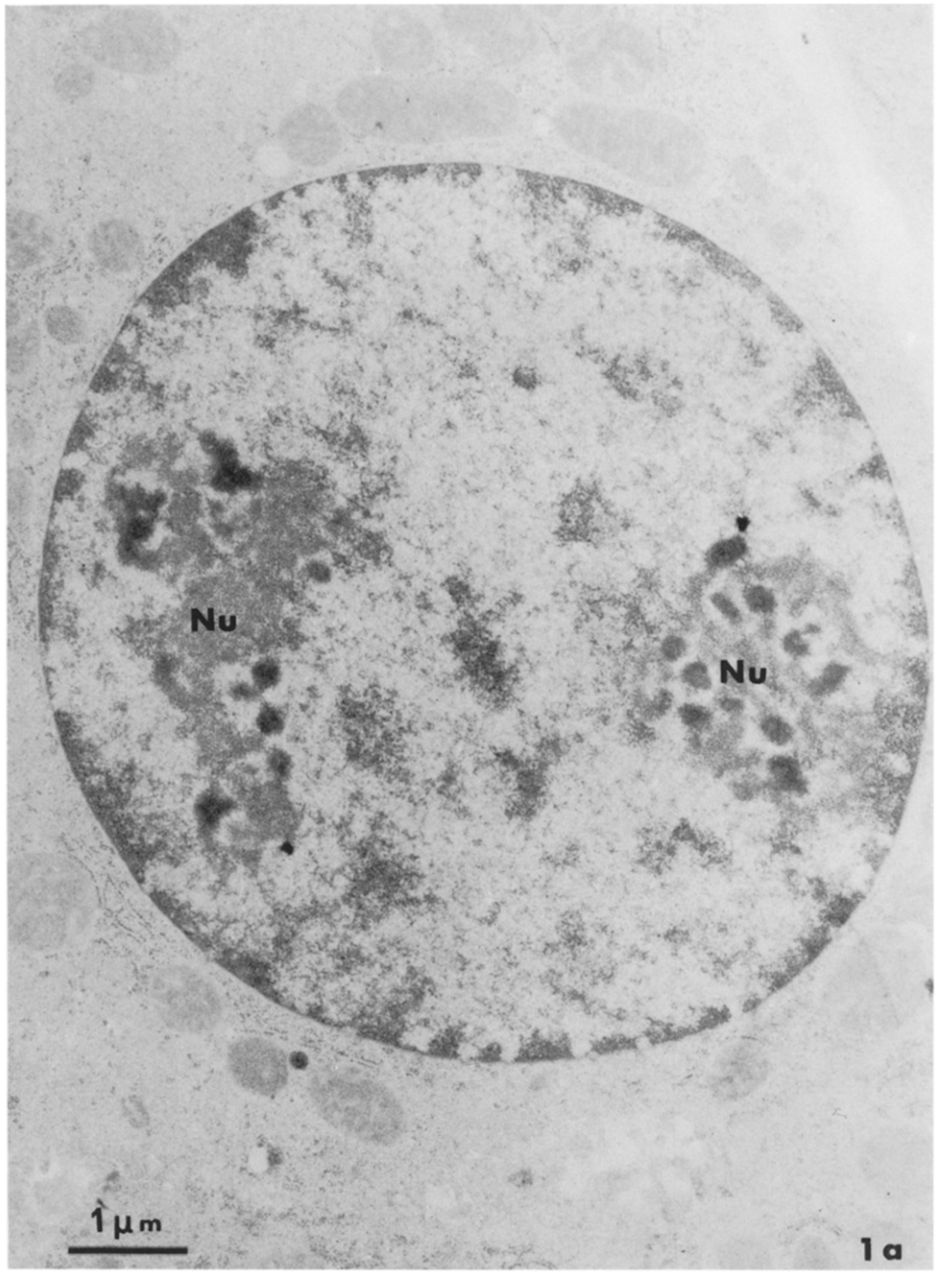
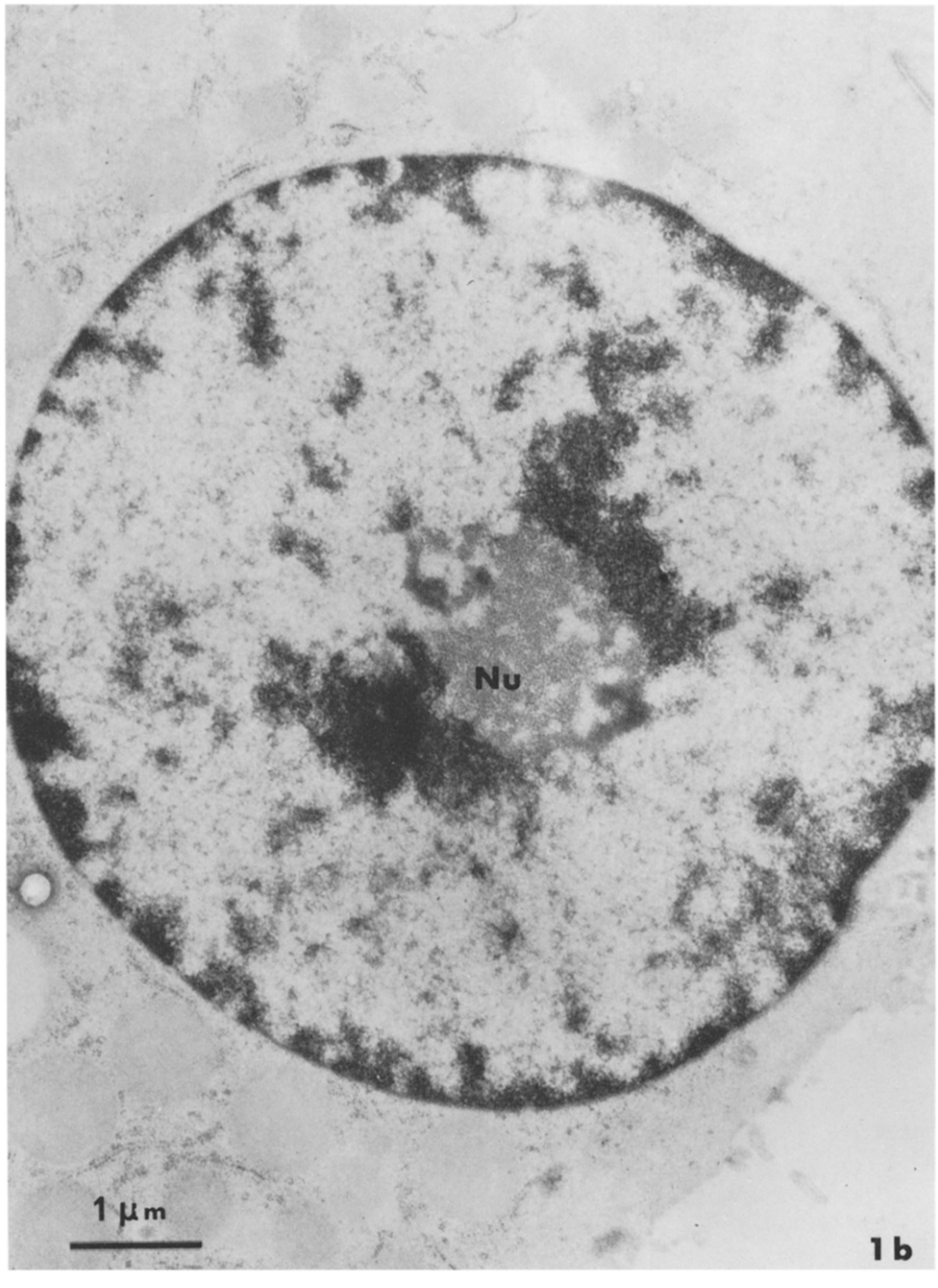


Fig. 1(*a*).



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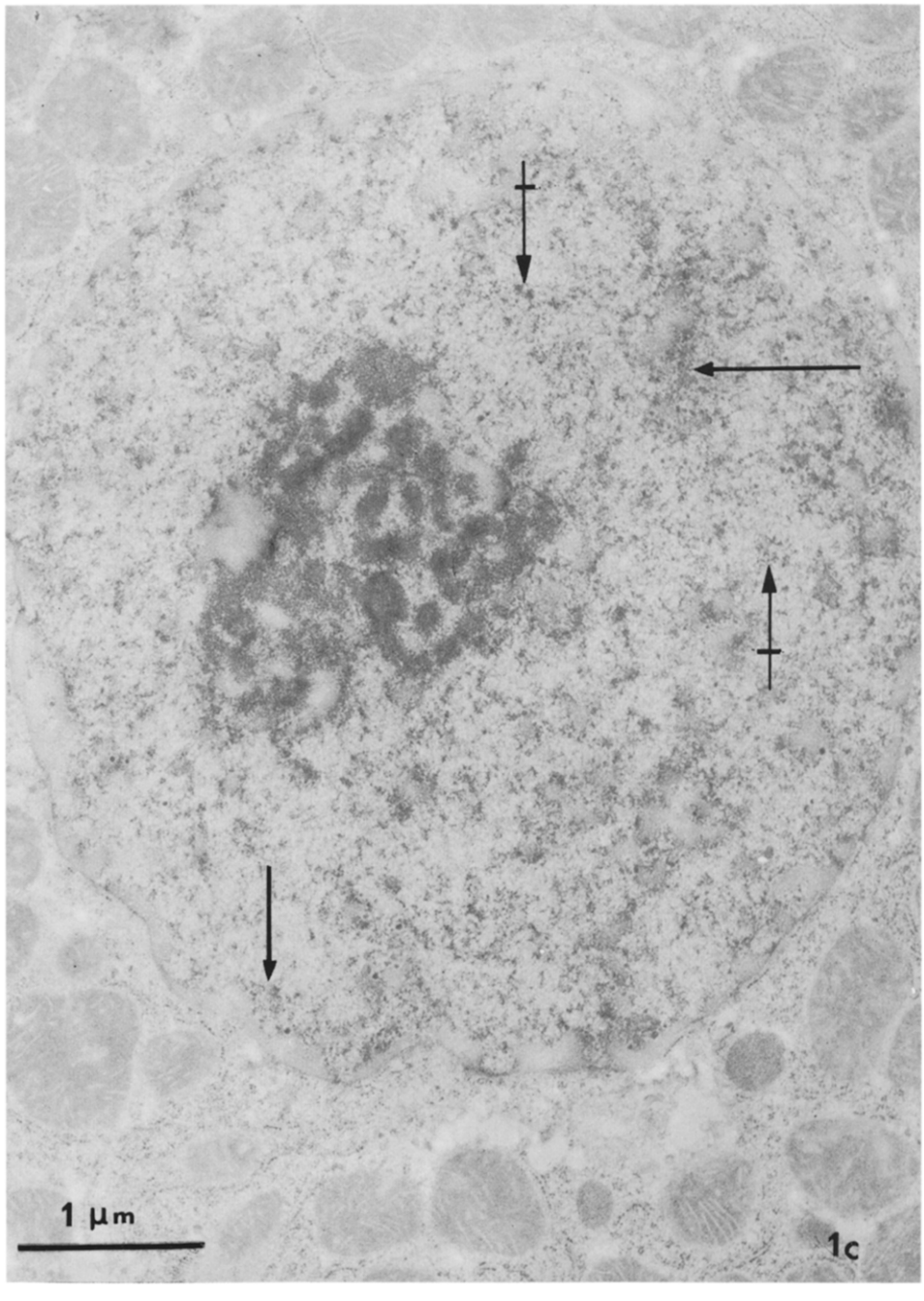

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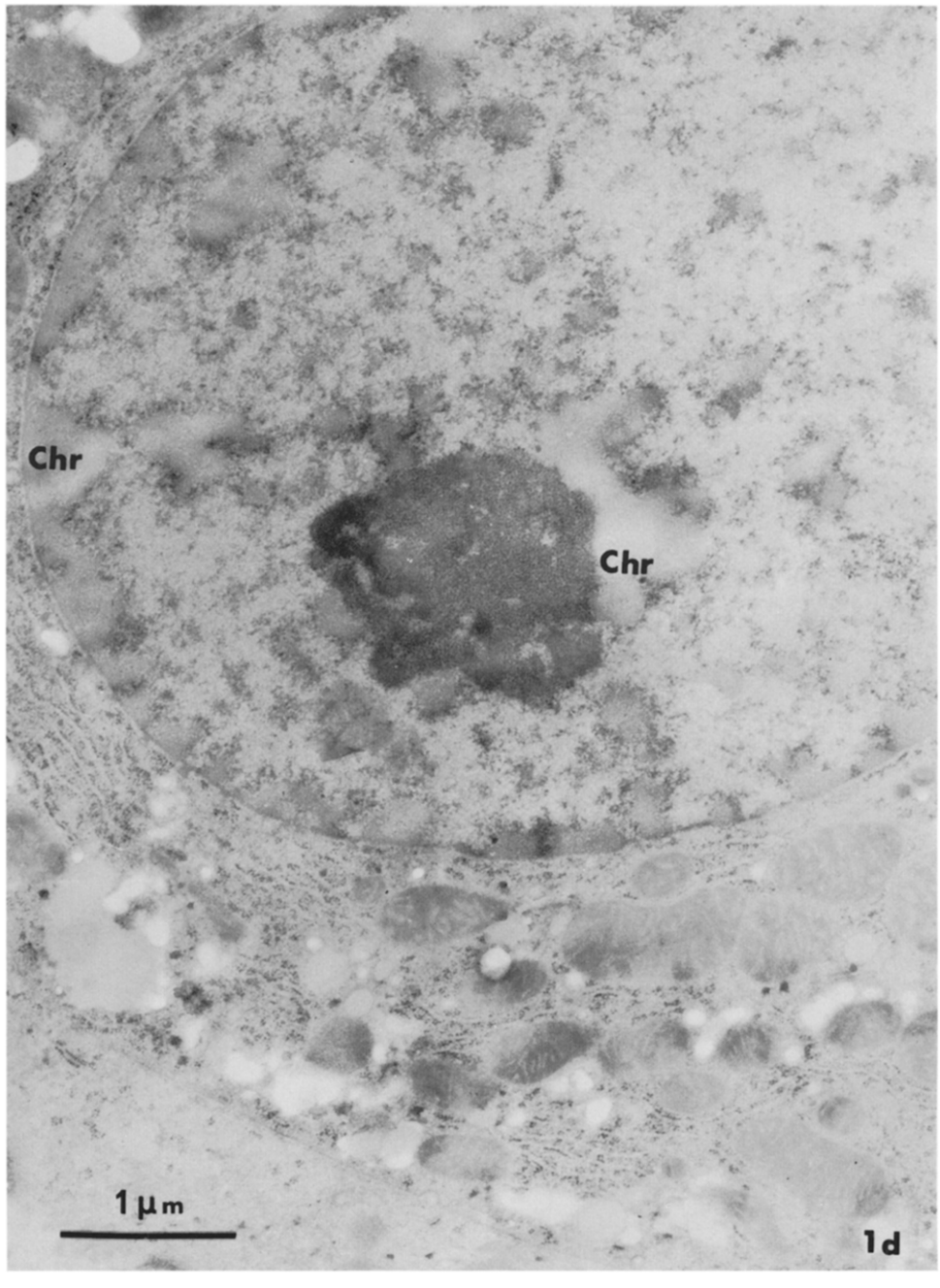

Fig. 1(*d*).

Fig. 1. *Nuclei of isolated rat liver cells.* (*a*) *Non-treated control cell. The nucleolus* (*Nu*) *exhibits classically intermingled strands of fibrillar and granular material. Uranyl acetate and lead citrate conventional stain.* (*b*) *Ten min treatment with* 9-OH E + (1 µg/ml). *The nucleolus is now compact and round-shaped. Uranyl and lead stain.* (*c*) *Non-treated control cell. RNP stain. The condensed chromatin has been bleached. Perichromatin* (⟶) *and interchromatin fibrils* (⟷) *are revealed.* (*d*) *Ten min treatment with* 9-OH E+(1 µg/ml). *RNP stain. The bleached chromatin is more abundant at the nuclear periphery and around the nucleolus. In the interchromatin space most interchromatin fibrils have disappeared, whereas perichromatin fibrils are less dense.*

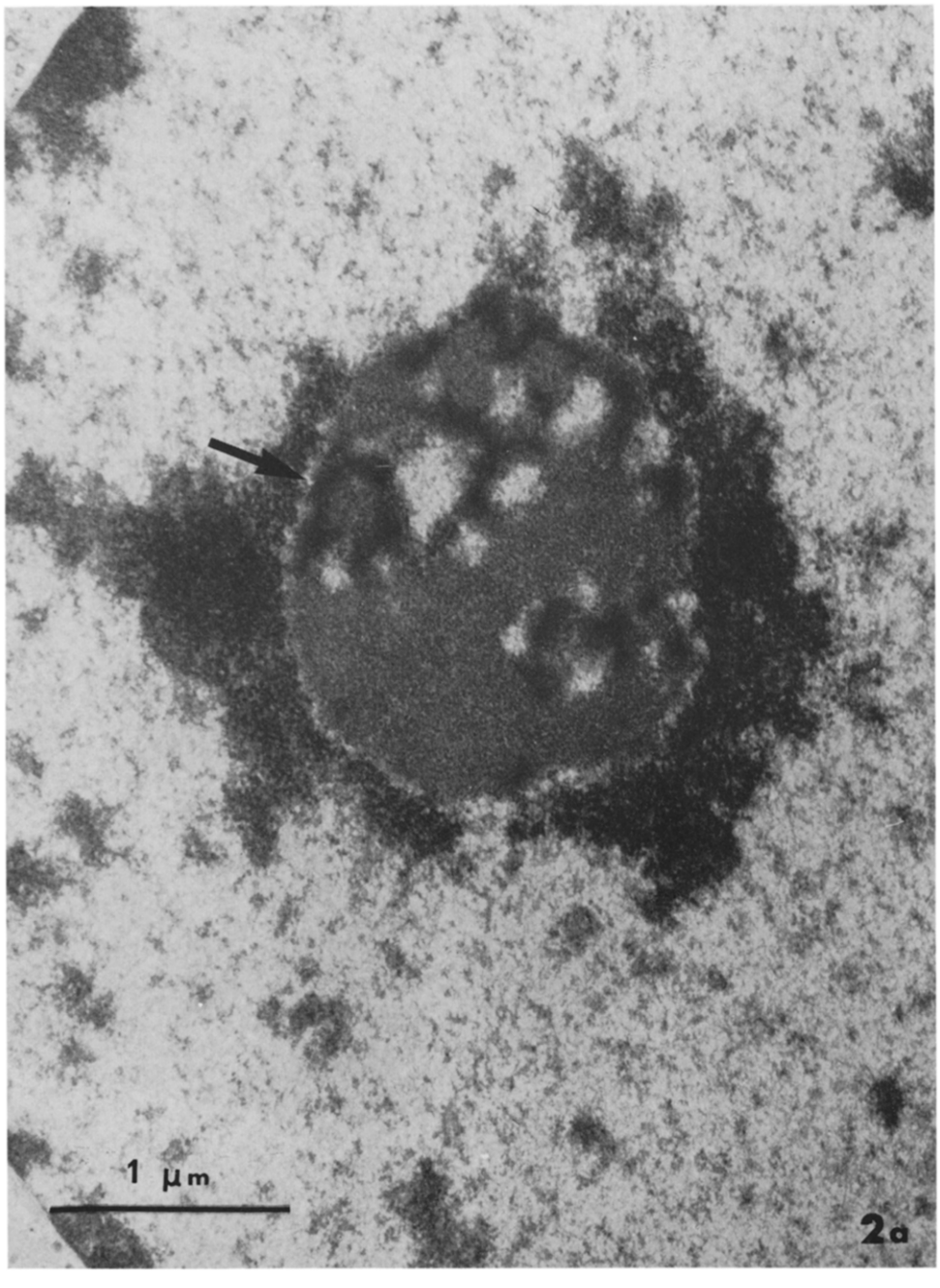

Fig. 2(*a*).

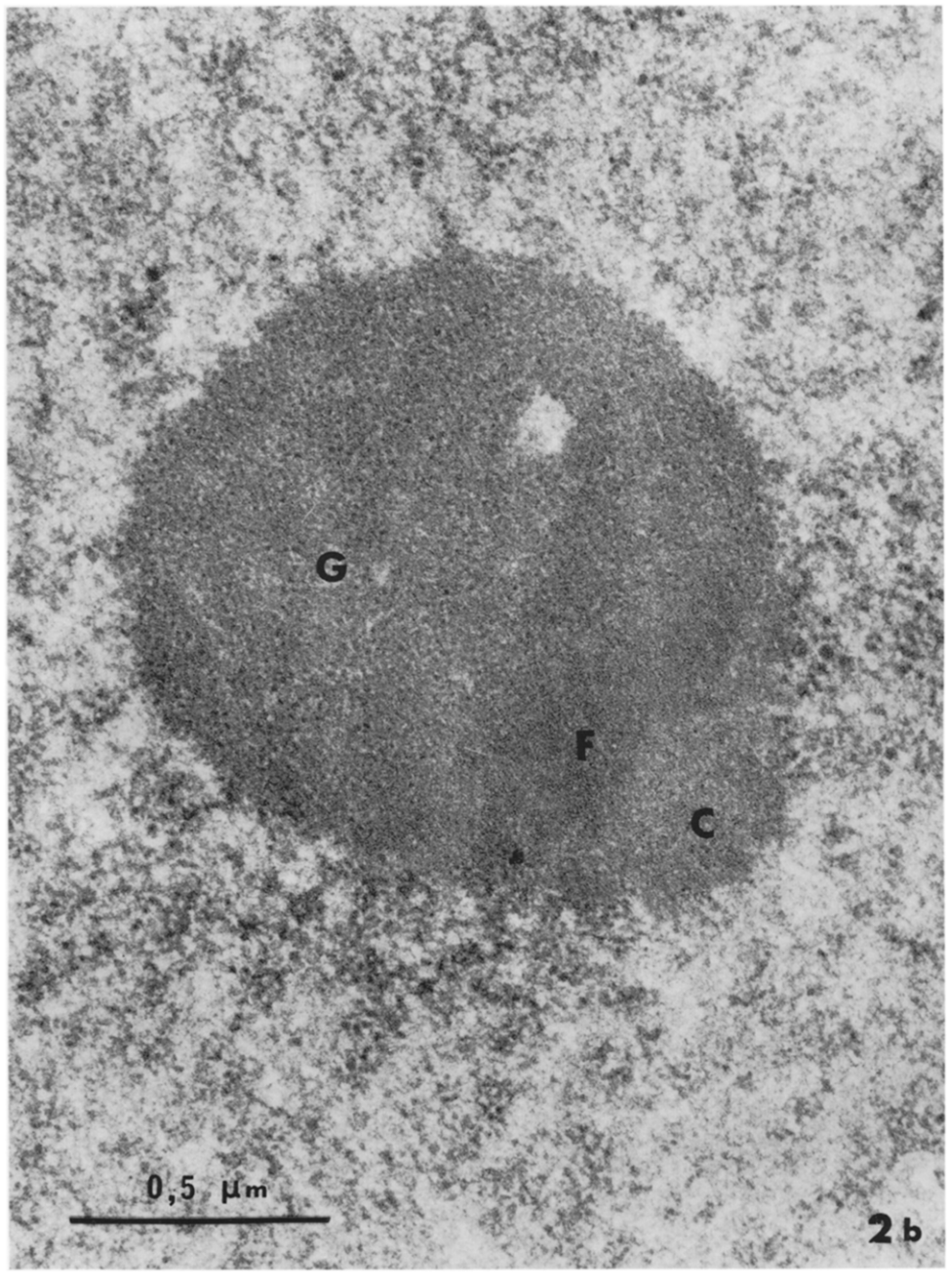

Fig. 2(*b*).

Fig. 2. *Nucleoli of isolated rat liver cells.* (*a*) *Two hr treatment with* 9-OH E+ (1 µg/ml). *Conventional staining reveals the presence of a thin rim of less condensed material* (*arrow*) *separating the nucleolar body from the surrounding condensed chromatin. This modification can also be observed after a* 10-*min treatment. A longer period of contact with the drug* (1 µg/ml) *has not resulted in further nuclear changes* (*compare with Fig.* 1*b*). (*b*) *Two hr treatment with* 9-OH E+ (5 µg/ml). *A typical nucleolar segregation occurs; it is characterized by the separation of nucleolar components into fibrils* (*F*), *granules* (*G*) *and a clear fibrillar area* (*C*). *Uranyl and lead stain.*

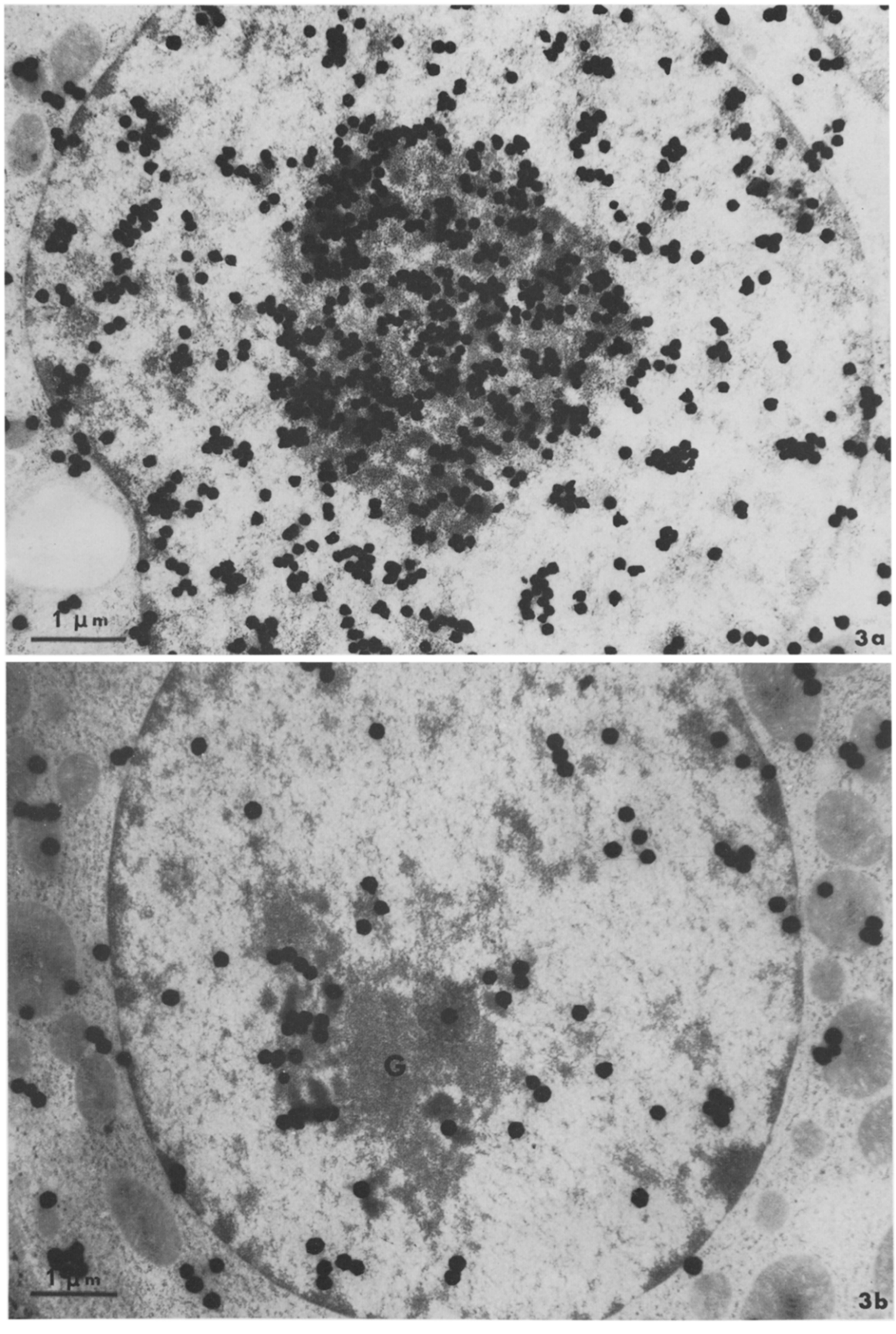

Fig. 3. Autoradiographs of isolated rat liver cells. (a) The cells have been labeled for 30 min with [5-^{3}H]-uridine in a drug-free medium. The nucleolus is labeled homogeneously over both its granular and fibrillar components. (b) The cells have been pretreated for 15 min with 9-OH E + (1 µg/ml), *then labeled for 30 min in the presence of the drug at the same concentration. The overall number of silver grains present in the nucleolar area is strongly reduced and there is a complete lack of labeling over the granular zone (G).*

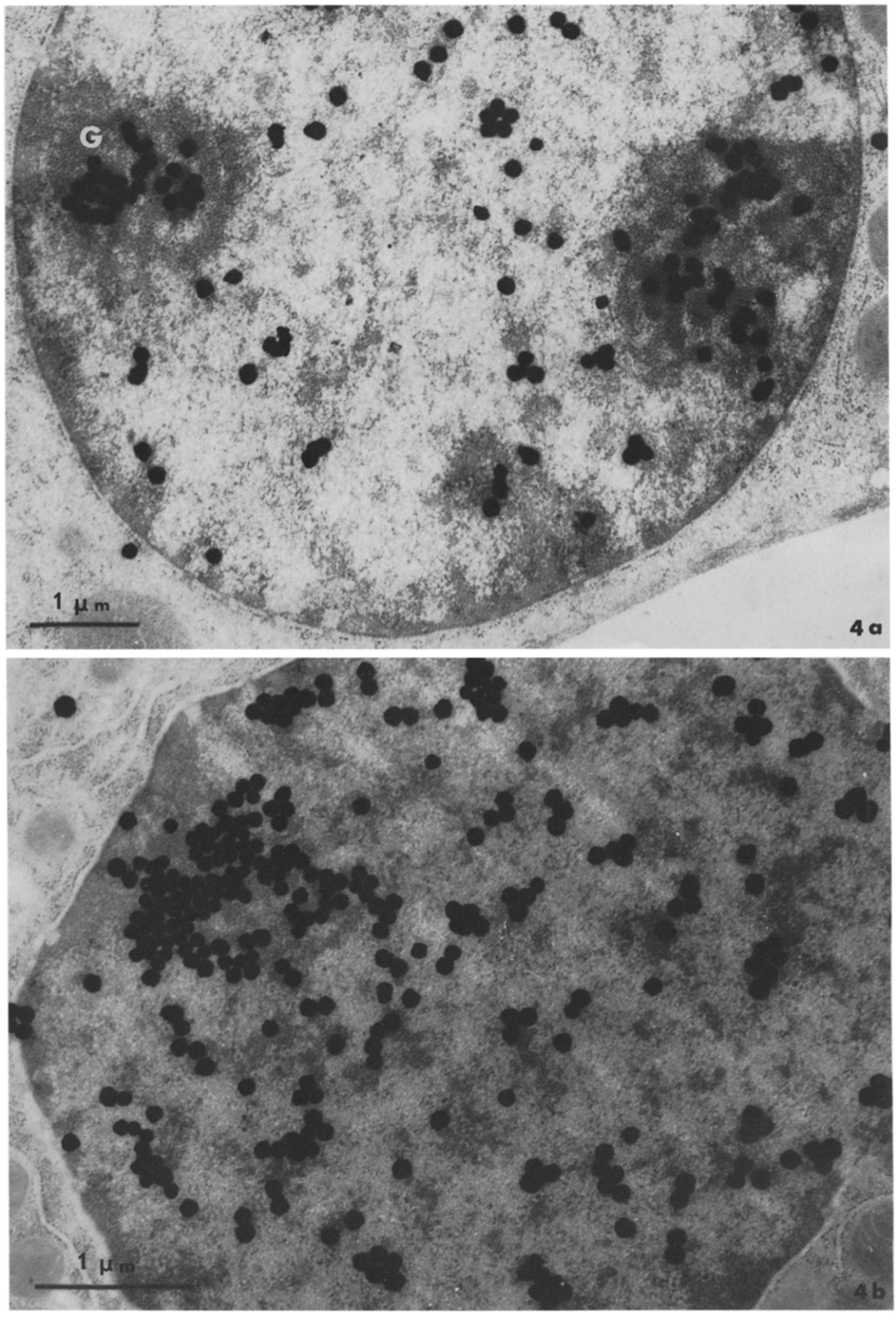

Fig. 4(a, b).

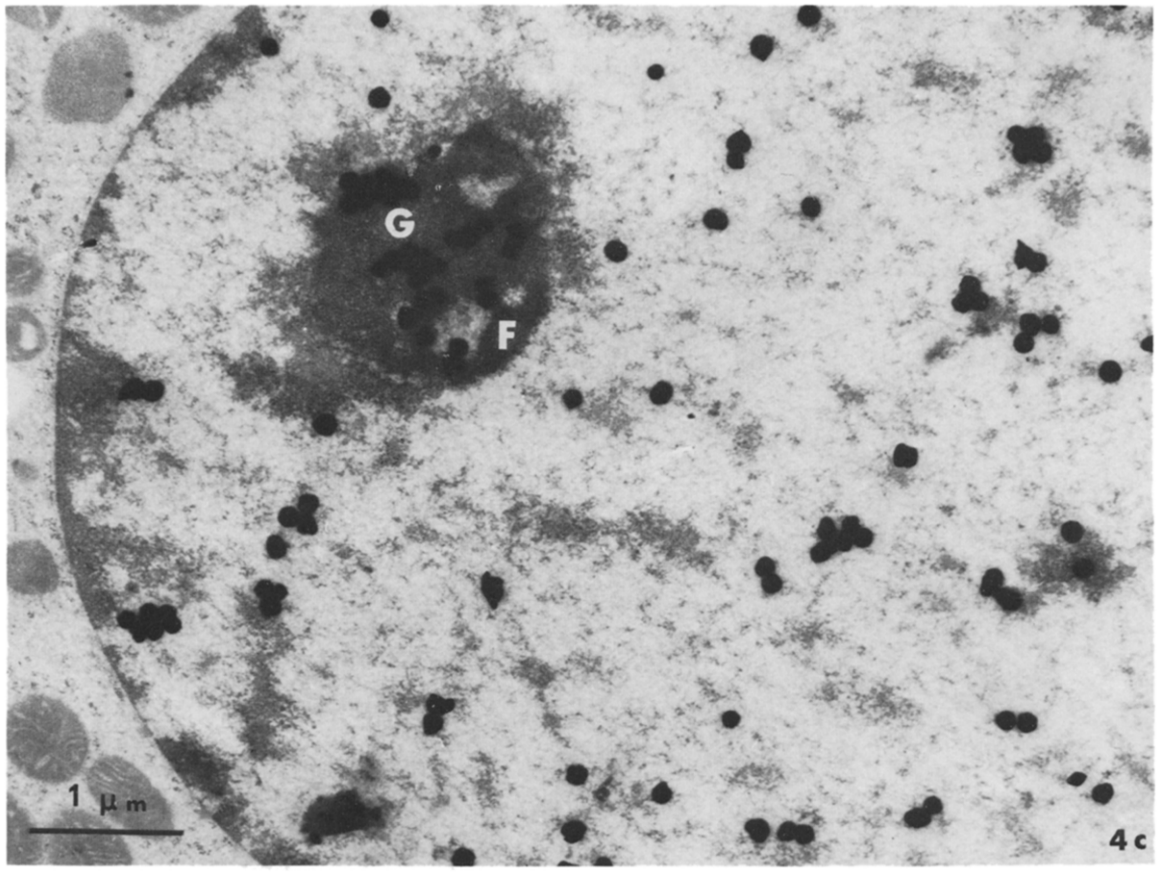

Fig. 4(*c*).

Fig. 4. *Autoradiographs of isolated rat liver cells.* (*a*) *Non-treated control cell. The hepatocytes have been labeled for* 15 *min then fixed immediately. Silver grains are present over the fibrillar zone of the nucleolus but not over the granular zone* (*G*). (*b*) *Non-treated cell. Fifteen min labeling followed by a* 1-*hr chase. The nucleolus is homogeneously labeled on its fibrillar and granular components.* (*c*) *Treated cell. The hepatocytes have been labeled as in Figs.* 4*a and* 4*b, followed by* 1-*hr chase in the presence of* 9-OH E + (1 µg/ml). *The overall labeling of the nucleolus is reduced, but there has been a shift of the radioactivity from the fibrillar* (*F*) *to the granular* (*G*) *component.*

RESULTS

Morphological alterations

Very similar morphological alterations are obtained with E, E+, 9-OH E and 9-OH E+, but 5- to 10-fold higher concentrations were necessary with compounds lacking the quaternary ammonium. So, only modifications due to 9-OH E+ will be described here.

Comparison of control cells (Fig. 1a) and cells treated with the ellipticine derivative at a dose of 1 μg/ml for only 10 min reveals that the drug induces a condensation of chromatin which appears more contrasted both along the nuclear envelope and around the nucleolar body (Fig. 1b). Clumping of chromatin is also visible within the faintly stained nucleoplasm. Following staining of only RNP with the method of Bernhard, nucleolar and extranucleolar RNP are revealed. The nucleoplasm of control cells contains many perichromatin fibrils at the border of the condensed chromatin and interchromatin fibrils within the interchromatin space (Fig. 1c). In ellipticine-treated cells, perichromatin fibrils adjacent to the condensed chromatin clumps are less abundant, whereas interchromatin fibrils have almost completely disappeared in the interchromatin space (Fig. 1d). The nucleolus of treated cells is more compact and has a rounded shape. The separation of fibrillar and granular nucleolar RNP is already visible and, generally, fibrils form dense strands clearly distinct from the granular zones. In most cases, the nucleolar body is separated from the surrounding condensed chromatin by a thin rim of less condensed material (Fig. 2a). Longer periods of contact with ellipticine at the same dose do not result in further nuclear structural changes. However, at a dose of 5 μg/ml, the lesions described above evolve within 2 hr towards a complete and typical nucleolar segregation characterized by the separation of nucleolar components into three distinct zones: fibrils, granules and clear fibrillar area (Fig. 2b). This is followed very rapidly by cell death.

Autoradiographic observations

The quantitative determinations of [5-^{3}H]-uridine incorporation in the two nucleolar and extra-nucleolar areas of the nuclei followed by statistical analysis demonstrates no statistically significant difference between 15-min and 1-hr pretreated cells. On the other hand, a highly significant difference in silver grain densities (risk less than 0.01, as estimated by *t* test) exists between either of these treated groups and the untreated controls. The labeling of the nucleolar area is strongly reduced in treated cells (73 and 80% after 15 and 60 min respectively). In the extra-nucleolar area, the decrease of the incorporation of [5-^{3}H]-uridine is of 47 and 57% respectively (Table 1).

Table 1. Quantitative autoradiography: incorporation of [5-^{3}H]-uridine in hepatocyte nuclei

	Nucleolar area	Extranucleolar area
Controls	36.89 ± 5.1*	7.87 ± 0.9
15 min 9-OH E+	10.86 ± 1.5	3.39 ± 0.4
1 hr 9-OH E+	7.08 ± 1.9	4.16 ± 0.4

*Silver grain densities are expressed in grains per square μm—mean ± S.D.

Figure 3a represents a normal control nucleus labeled for 30 min with [5-^{3}H]-uridine. Radioactivity is dispersed all over the nucleoplasm. The nucleolus is homogeneously labeled over fibrillar and granular components. On the other hand, in the cells pre-treated either for 15 min or 1 hr with 9-OH E+ (1 μg/ml) followed by 30 min of labeling the silver grains are present exclusively over the fibrillar nucleolar strands; the granular component is not labeled (Fig. 3b).

Contrary to the above results, the comparison of autoradiograms of hepatocytes after a 15-min pulse with [5-^{3}H]-uridine followed by a 1-hr or a 3-hr chase with or without 9-OH E+ does not reveal any difference in the localization of silver grains in the nucleoli of treated and untreated cells. At the end of the 15-min period of labeling, the association of the radioactivity with the fibrillar elements of the nucleoli is evident (Fig. 4a). Following 1 or 3 hr of subsequent post-incubation, the nucleoli of control cells exhibit a progressive migration of the radioactivity from the fibrillar to the granular compartment (Fig. 4b).

When the chase is carried out in the presence of 9-OH E+, a moderate reduction of the density of silver grains is observed over the extra-nucleolar and nucleolar areas. In the nucleolus, a shift of the radioactivity from the fibrillar towards the granular compartment takes place (Fig. 4c).

DISCUSSION

Our observations of structural and functional nuclear lesions induced by an ellipticine derivative in isolated rat liver cells must be discussed in relation to our recent knowledge of the significance of nuclear RNP components and on the basis of biochemical data dealing with ellipticine action on cellular metabolism.

Quantitative high resolution autoradiography has shown a rapid decrease of [5-^{3}H]-uridine incorporation with moderate selectivity for nucleolar incorporation. This agrees well with biochemical determination of Kann and Kohn [19] carried out on leukemia L 1210 cells. Our results can therefore be interpreted as reflecting an inhibition of nucleolar and extranucleolar RNA synthesis, though the hypothesis of a drug-related effect on uridine uptake or phosphorylation cannot be completely excluded.

From a structural point of view, the functional disturbances were associated with the segregation of nucleolar components and induced the disappearance of perichromatin fibrils and interchromatin fibrils.

Nucleolar segregation characterized by the coalescence of nucleolar RNP components into distinct zones has been extensively studied [20]. From the studies of Simard [21] and of Simard and Bernhard [22], it is now well-established that this nucleolar lesion can be considered as specific for the action of drugs binding to DNA and affecting the template activity of ribosomal DNA. The agents known to induce nucleolar segregation include intercalating drugs [22]. However, in the case of ellipticine, relatively high doses (5 μg/ml) which lead rapidly to cell death were required to induce a complete segregation of nucleolar components similar to that caused by actinomycin D. This might be imputable to the relatively low selectivity of ellipticine binding to rDNA, and confirms that nucleolar segregation is a dose-dependent lesion [20].

Several recent studies have demonstrated that perichromatin fibrils formed initially at the border of the condensed chromatin contain the newly synthesized heterogeneous nuclear RNA [14] and that they migrate subsequently towards the interchromatin space [23]. Thus, the almost complete disappearance of perichromatin and interchromatin fibrils, which occurred simultaneously with the condensation of chromatin as rapidly as 15 min after the initiation of the ellipticine treatment, can be considered as reflecting the inhibition of 43% of uridine uptake in the nucleoplasm.

Since the earlier works of Granboulan and Granboulan [24], later on confirmed by many investigators [14, 25], it is well-established that the fibrillar RNP component of the nucleolus is labeled first following a short incubation time with [5-^{3}H]-uridine and that the subsequent shift of radioactivity from the fibrils to the nucleolar granules represents the structural counterpart of pre-rRNA processing. *Therefore, the selective and persistent accumulation of the radioactivity over the nucleolar fibrils following different labeling periods with* [5-^{3}H]-*uridine of ellipticine-treated cells represents a block of pre-rRNA processing.*

Similar effects have been reported under the action of unrelated drugs such as cordycepin [26] and camptothecin [27]. In both cases, the inhibition of pre-rRNA processing was related to the formation of shortened pre-rRNA molecules which could not be normally processed. According to Kann and Kohn [19], such pre-rRNA chain shortening does not occur in the presence of ellipticine, due probably to the rapid reversibility of the complex formed by this drug with DNA. Snyder *et al.* [28] proposed that the inhibition of the delay in the onset of the pre-rRNA processing induced by ellipticine might be related to a direct binding of the drug at hypothetical sites of these RNA molecules.

However, our observations differ from those of these authors since we observed in isolated rat liver cells that the processing of the normally synthesized pre-rRNA was not blocked during post-incubation in the presence of ellipticine. Thus, the possibility that the inhibition of the processing of pre-rRNA synthesized in the presence of the drug might be secondary to the inhibition of heterogeneous nuclear RNA synthesis cannot be excluded. Such an effect was proposed by Granick [29] to account for the partial inhibition of pre-rRNA processing and the further degradation of pre-ribosomes in nucleoli of chick fibroblasts treated with the adenosine analogue, 5-6 dichloro 1-βD-ribofuranosyl benzimidazole (DRB).

Furthermore, as shown by Geuskens and Bernhard [30] with actinomcyin D, nucleolar segregation still permits the normal processing of pre-rRNA synthesized prior to the action of the antibiotic. Hadjiolov and Nikolaev [31] generalized these observations and postulated that agents inducing nucleolar segregation have the particularity of blocking pre-rRNA transcription without affecting pre-rRNA processing.

Another point to be considered is the absence in ellipticine-treated cells of newly formed perichromatin granules. It was indeed reported in several studies [26, 27, 32–35] that drugs blocking totally or partially pre-rRNA processing induced the appearance of perichromatin granules at the level of intra and perinucleolar chromatin. Surprisingly, such granules were never observed following ellipticine treatment. Since, as discussed above, E does not induce pre-rRNA shortening, this finding indirectly supports our initial hypo-

thesis (see Puvion and Moyne [36] for a review) that the nucleolar perichromatin granules do represent degradation forms of abnormally shortened pre-rRNA.

Acknowledgements—The authors are indebted to Dr. W. Bernhard (I.R.S.C., Villejuif) and to Prof. C. Paoletti (Laboratoire de Chimiothérapie Expérimentale, I.G.R., Villejuif), who initiated this work.

They thank Prof. C. Paoletti for providing ellipticine derivatives, Prof. E. Leduc (Brown University, Providence) for critical reading of the manuscript and Mrs J. Burglen for technical assistance.

REFERENCES

1. FESTY B, POISSON J, PAOLETTI C. A new DNA intercalating drug: methoxy-9-ellipticine. *FEBS Lett* 1971, **17**, 321–325.
2. KOHN KW, WARING MJ, GLAUBIGER D, FRIEDMAN CA. Intercalative binding of ellipticine NSC-71795 to DNA. *Cancer Res* 1975, **35**, 71–76.
3. BERNHARD W. A new staining procedure for electron microscopical cytology. *J Ultrastruct Res* 1969, **27**, 250–267.
4. DALTON LK, DEMERAC S, ELMES BC, LODER JW, SWAN JM, TEITEI T. Synthesis of the tumor-inhibitory alkaloids, ellipticine, 9-methoxy ellipticine and related pyrido (4-3,b) carbazoles *Aust J Chem* 1967, **20**, 2715–2722.
5. HARTWELL JL, ABBOTT BJ. Antineoplastic principles in plants. Recent developments in the field. *Adv Pharmacol Chemother* 1969, **7**, 117–209.
6. LEMEN J, HAYAT M, MATHÉ G *et al.* Methoxy-9-ellipticine lactate. I—Experimental study (oncostatic and immuno-suppressive actions; preclinical pharmacology). *Rev Eur Et Clin Biol* 1970, **15**, 534–538.
7. MATHÉ G, HAYAT M, DE VASSAL F *et al.* Methoxy-9-ellipticine lactate. III—Clinical screening: its action in acute myeloblastic leukemia. *Rev Eur Et Clin Biol* 1970, **15**, 541–545.
8. LE PECQ JB, GOSSE C, DAT-XUONG N, PAOLETTI C. Un nouveau composé antitumoral:l'hydroxy-9-ellipticine. Action sur la leucémie L 1210 de la souris. *C R Acad Sci [D] (Paris)* 1973, **277**, 2289–2291.
9. LE PECQ JB, GOSSE C, DAT-XUONG N, PAOLETTI C. Deux nouveaux dérivés anti-tumoraux:l'hydroxy-9-méthyl-2-ellipticinium et l'hydroxy-9-diméthyl-2-6-ellipticinium. Action sur la leucémie L 1210 de la souris. *C R Acad Sci [D] (Paris)* 1975, **281**, 1365–1367.
10. LE PECQ JB, GOSSE C, DAT-XUONG N, CROS S, PAOLETTI C. Anti-tumor activity of 9-hydroxy ellipticine NSC210717 on L 1210 mouse leukemia and the effect of route of injection. *Cancer Res* 1976, **36**, 3067–3076.
11. PAOLETTI C, CROS S, DAT-XUONG N, LECOINTE P, MOISAND A. Comparative cytotoxic and antitumoral effects of ellipticine derivatives on mouse L 1210 leukemia. *Chem Biol Interact* 1979, **25**, 45–58.
12. DUFER J, DESPLACES A, AUROUSSEAU M. Cytochemical study of rat leukocytes treated by methoxy-9-ellipticine. *Ann Pharm Fr* 1973, **31**, 441–450.
13. OUSTRIN ML, PIERAGGI MT. Action des hydroxy-9 et methoxy-9 ellipticines sur la cellule HeLa S_3 on culture "in vitro". *C R Acad Sci [D] (Paris)* 1974, **278**, 1967–1970.
14. FAKAN S, PUVION E. The ultrastructural visualization of nucleolar and extranucleolar RNA synthesis and distribution. *Int Rev Cytol* 1980, **65**, 255–299.
15. PUVION E, GARRIDO G, VIRON A. Technique rapide d'isolement et de survie en monocouches d'hépatocytes de rats adultes. *C R Acad Sci [D] (Paris)* 1974, **279**, 509–512.
16. HAASE G, JUNG G. Herstellung von Einkorn-schichten aus photographischen emulsionen. *Naturwissenschaften* 1964, **51**, 404–405.
17. BOUTEILLE M, DUPUY-COIN AM, MOYNE G. Techniques of localization of proteins and nucleoproteins in the cell nucleus by high resolution autoradiography and cytochemistry. In: O'MALLEY BW, HARDMAN JG, eds. *Methods in Enzymology.* New York, Academic Press, 1975, **40**, 3–41.
18. FAKAN S, BERNHARD W. Localization of rapidly and slowly labeled nuclear RNA as visualized by high resolution autoradiography. *Exp Cell Res* 1971, **67**, 129–141.
19. KANN HE, KOHN KW. Effects of deoxyribonucleic acid-reactive drugs on ribonucleic acid synthesis in leukemia L 1210 cells. *Mol Pharmacol* 1972, **8**, 551–560.
20. SIMARD R, LANGELIER Y, MANDEVILLE R, MAESTRACCI N, ROYAL A. Inhibitors as tools in elucidating the structure and function of the nucleus. In: BUSCH H, ed. *The Cell Nucleus.* New York, Academic Press, 1974 Vol. III, 447–487.

21. SIMARD R. Specific nuclear and nucleolar ultrastructural lesions induced by proflavin and similarly acting anti-metabolites in tissue culture. *Cancer Res* 1966, **26**, 2316–2328.
22. SIMARD R, BERNHARD W. Le phénomène de la ségrégation nucléolaire: spécificité d'action de certains anti-métabolites. *Int J Cancer* 1966, **1**, 463–479.
23. PUVION E, MOYNE G. Intranuclear migration of newly synthesized extra-nucleolar ribonucleoproteins. A high resolution quantitative autoradiographical and cytochemical study. *Exp Cell Res* 1978, **115**, 79–88.
24. GRANBOULAN N, GRANBOULAN P. Cytochimie ultrastructurale du nucléole. II. Etude des sites de synthèse du RNA dans le nucléole et le noyau. *Exp Cell Res* 1965, **38**, 604–619.
25. FAKAN S. High resolution autoradiography studies on chromatin functions. In: BUSCH H, ed. *The Cell Nucleus, Chromatin—Part B.* New York, Academic Press, 1978, Vol. V, 3–53.
26. PUVION E, MOYNE G, BERNHARD W. Action of 3′ deoxyadenosine (cordycepin) on the nuclear ribonucleoproteins of isolated liver cells. *J Microsc Biol Cell* 1976, **25**, 17–32.
27. GAJKOWSKA B, PUVION E, BERNHARD W. Unusual perinucleolar accumulation of ribonucleoprotein granules induced by camptothecin in isolated liver cells. *J Ultrastruct Res* 1977, **60**, 335–347.
28. SNYDER AL, KANN HE, KOHN KW. Inhibition of the processing of ribosomal precursor RNA by intercalating agents. *J Mol Biol* 1971, **58**, 555–565.
29. GRANICK D. Nucleolar necklaces in chick embryo fibroblast cells. *J Cell Biol* 1975, **65**, 389–417.
30. GEUSKENS M, BERNHARD W. Cytochimie ultrastructurale du nucléole. III. Action de l'actinomycine D sur le métabolisme du RNA nucléolaire. *Exp Cell Res* 1966, **44**, 579–598.
31. HADJIOLOV AA, NIKOLAEV N. Maturation of ribosomal ribonucleic acids and the biogenesis of ribosomes. *Prog Biophys Mol Biol* 1976, **31**, 95–144.
32. RECHER L, CHAN H, BRIGGS L, PARRY N. Ultrastructural changes inducible with the plant alkaloid camptothecin. *Cancer Res* 1972, **32**, 2495–2501.
33. DERENZINI M, MOYNE G. The nucleolar origin of certain perichromatin-like granules. A study with α amanitin. *J Ultrastruct Res* 1978, **62**, 213–219.
34. PUVION E, BACHELLERIE JP, BURGLEN MJ. Nucleolar perichromatin granules induced by dichlorobenzimidazole-riboside. *J Ultrastruct Res* 1979, **69**, 1–12.
35. VAZQUEZ-NIN G, ECHEVERRIA OM, PEDRON J. Ultrastructural and autoradiographic study of the effects of bleomycin on the interphase nucleus of cultured normal cells. *Cancer Res* 1979, **39**, 4218–4223.
36. PUVION E, MOYNE G. *In situ* localization of RNA structures In: BUSCH H, ed. *The Cell Nucleus, Nuclear Particles—Part A.* New York, Academic Press, 1981, Vol. VIII, 59–116.