

RNA SYNTHESIS IN RAT AND MOUSE HEPATIC CELLS AS STUDIED WITH LIGHT AND ELECTRON MICROSCOPE RADIOAUTOGRAPHY

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Work on radioautographic localization of RNA synthesis has been reviewed by Prescott (1). The findings with this method show good correspondence with biochemical evidence by indicating the nucleus as the presumptive site of RNA synthesis, and the probability of transport of RNA from the nucleus to the cytoplasm has been established. The method has been applied by Nossal et al. (2) in semiquantitative studies related to RNA synthesis.

The effect of the hormone aldosterone on RNA synthesis has been studied radioautographically

in toad bladder tissue (3). An increase of uridine incorporation as a result of the hormone action was observed. Biochemical evidence has indicated a selective alteration of hepatic messenger RNA synthesis resulting from the action of several hormones, among them hydrocortisone (4).

It seemed desirable to use radioautography to look for a morphological expression of the events described by Kidson and Kirby (4). Thus, the events of RNA synthesis could be studied *in situ* with the light microscope, and radioautographs

TABLE I
Effect of Hydrocortisone on Radioautographic Grain Counts in Rat and Mouse Hepatic Cells

Animal	Nucleus		Cytoplasm	
	Mean	Sum of squares	Mean	Sum of squares
R Or(20 min)	5.4	146.7	10.5	197.7
R H(50 min) Or(20 min)	12.3	581.6	16.0	982.0
Difference	6.9		5.5	
Significance of difference	$t = 7.2$	$P < 0.01$	$t = 4.6$	$P < 0.01$
O Ur(20 min)	4.0	106.0	10.1	296.0
O H(50 min) Ur(20 min)	8.2	367.0	17.3	900.0
Difference	4.2		7.2	
Significance of difference	$t = 5.7$	$P < 0.01$	$t = 6.2$	$P < 0.01$
O Or(20 min)	6.5	233.5	13.0	539.0
O H(50 min) Or(20 min)	10.0	261.0	17.5	1127.5
Difference	3.5		4.5	
Significance of difference	$t = 4.6$	$P < 0.01$	$t = 3.2$	$P < 0.01$

The abbreviations used in this table are O, C₂₀ mouse; R, R(Amsterdam) rat; Or, orotic acid-5-H³; Ur, uridine-G-H³; and H, hydrocortisone.

made under sufficiently standardized conditions would make possible semiquantitative measurements by means of grain counts. For the exact localization of the label in the cells, high resolution radioautography with the electron microscope on ultrathin sections could be used.

In the present investigation, RNA labeling was done by means of either tritiated orotic acid or uridine, with relatively short labeling times. In this paper, the labeling time is understood as the time elapsed between the intraperitoneal injection of the label and the fixation of tissue in osmium tetroxide.

We have attempted to answer the following questions:

1. What is the exact localization of grains in the nucleus and cytoplasm as seen with high resolution radioautography?
2. What is the effect of varying the labeling time (4 intervals between 10 and 80 min) on the grain counts in the nucleus and/or the cytoplasm of hepatic cells?
3. What is the effect on the grain counts in the hepatic nucleus and/or the cytoplasm if hydrocortisone is administered prior to the labeled compound?

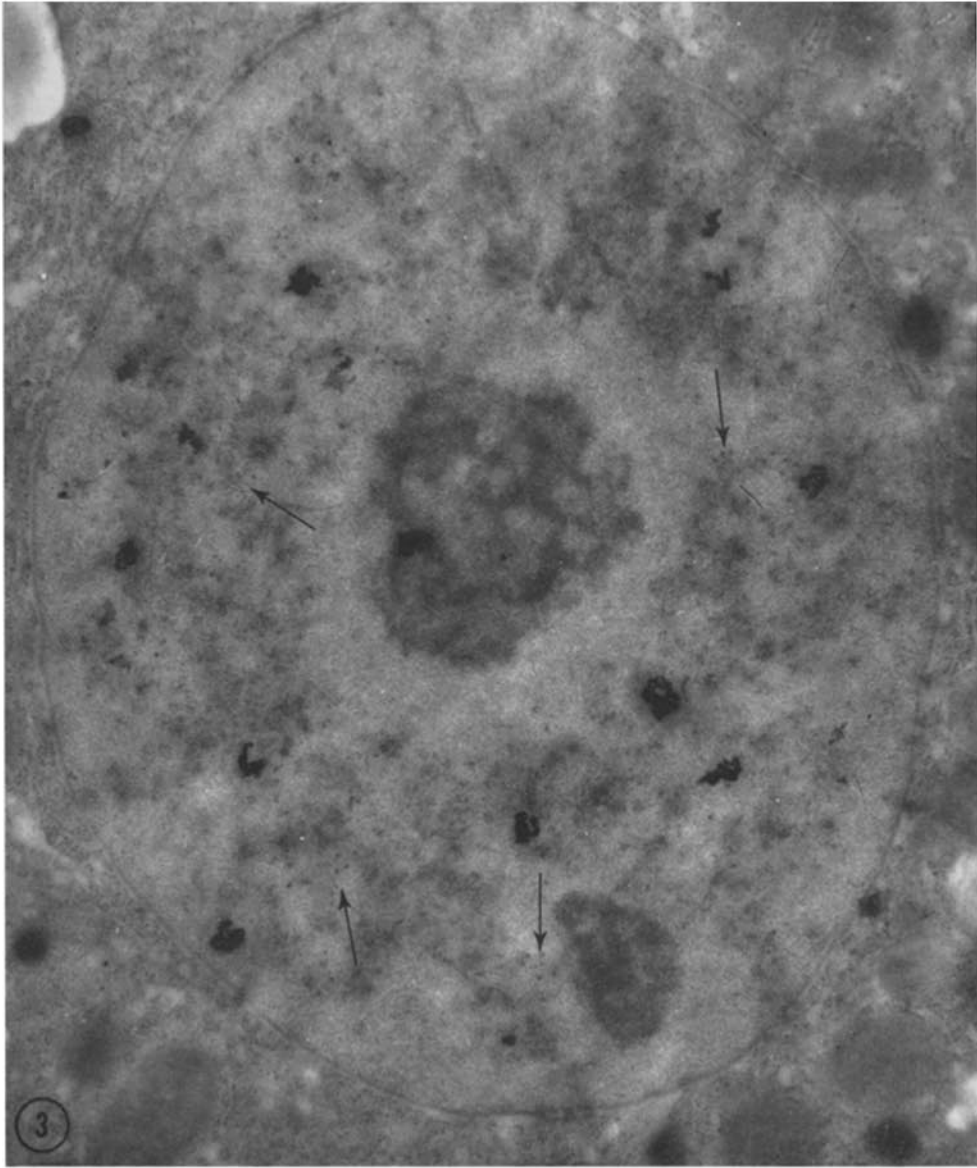
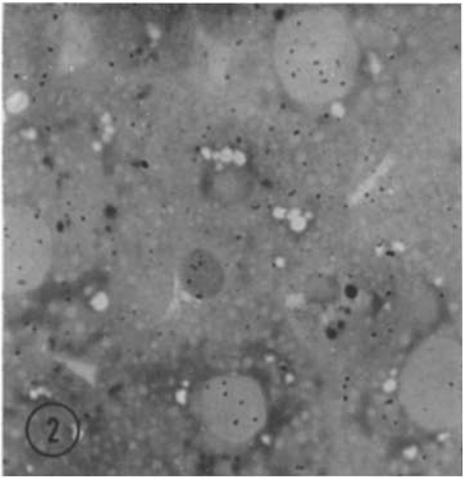
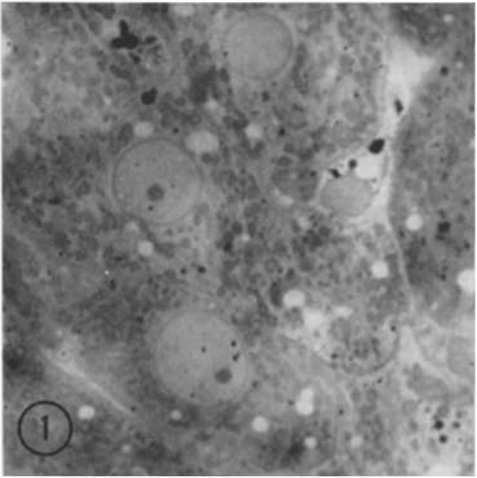
MATERIAL AND METHODS

R(Amsterdam) inbred strain female rats aged about 2 months and weighing 125 to 150 g were used, as

FIGURES 1 and 2 Light microscope radioautographs of 0.9 μ Epon sections of mouse liver showing effect of hydrocortisone treatment on the number of grains over nucleus and cytoplasm.

FIG. 1, untreated animal; FIG. 2, hydrocortisone-treated mouse. $\times 1200$.

FIGURE 3 Electron microscope radioautograph of a hepatic cell of a hydrocortisone-treated rat. In the nucleus, grains occur over the nucleolus and over areas that are distinguishable from densely packed finely granular chromatin. Perichromatinic granules (arrows). $\times 9000$.



well as O₂₀ female mice weighing 20 g and aged about 2 months, and C₅₇Bl mice, kindly provided by The Netherlands Cancer Institute, Amsterdam.

To answer the three questions posed in the introduction, we grouped and coded the experimental animals as shown in Table I: rats, R; O₂₀ mice, O. The label used, i.e. uridine (Ur) and orotic acid (Or), is indicated after the animal code symbol. If treatment with hydrocortisone (H) preceded the injection of the label, this is indicated before the label code symbol. The duration of treatment and labeling times are indicated between parentheses and comprise the total treatment time up to the animal's death.

Animals were given a standard laboratory diet and tap water ad lib. All experiments were done at the same time of the day (between 11.00 and 12.00 a.m.) in order to exclude diurnal variations. In the experiments on the effect of hydrocortisone on RNA synthesis, hydrocortisone acetate (Organon, Oss, The Netherlands) was injected intraperitoneally in a dosage of either 10 mg (rats) or 2.5 mg (mice). Some of the animals were injected intraperitoneally with orotic acid-5-H³ (specific activity, 2300 to 4000 mc/mmole, Radiochemical Centre, Amersham, England) in a dosage of 1 mc (rats) or 0.25 mc (mice). In addition, three of the O₂₀ mice were injected intraperitoneally with 0.25 mc of uridine-G-H³ (specific activity, 1760 mc/mmole, Radiochemical Centre).

The animals were killed by decapitation. Tissue processing consisted of cutting small (approximately 1 mm³) blocks from the left lobe of the liver, followed by fixation for 1 hr in a mixture of two parts osmium tetroxide (2%) and 1 part collidine buffer (pH 7.2) (5). After dehydration in alcohol, the blocks were embedded in Epon 812 (6). Sectioning was done on a Porter-Blum MT-2 microtome set for 0.9 μ. The sections were mounted on glass slides. For both light and electron microscope radioautography, Ilford L-4 emulsion (10 g diluted in 20 ml distilled water) prepared by the loop method according to Caro and van Tubergen (7) was used. It was essential for the comparison of grain counts in differently treated animals that the various sets of slides be coated with the same emulsion prepared in one session. After a constant exposure time of 10 days at 4°C, radioautographs were developed under standard conditions in a D 19 developer for 2 min at 20°C and fixed in Kodak rapid fixer AM 33 for 2 min. After rinsing, the slides were dried and stained with toluidine blue (1%) in borax (1%) for 3 min, rinsed, and dried.

The following method was used for grain counting. In several sections, the observer counted the grains occurring in the nucleus and/or cytoplasm of 30 randomly chosen cells whose nuclear and cell diameters, as measured with a micrometer, were about 8 and 25 μ, respectively. The reliability of the method

was checked by comparison of several counts from different parts of the same tissue.

The standard deviation of the arithmetical means of samples and the significance of the differences between arithmetical means of samples were tested according to methods described by Fisher (8).

From the blocks of some of the animals, ultra thin sections were made for high-resolution radioautography. The methods used for this purpose have already been described (9). These sections were stained with lead cacodylate (10).

Paraffin sections prepared from liver tissue of some of the animals fixed in 1% buffered formaldehyde were treated with RNASE A (Bovine pancreas, Sigma Chemical Co., St. Louis, Missouri) in a concentration of 1 mg/ml of distilled water. These sections were incubated for 2 hr at either 37° or 65°C, and then compared with control paraffin sections kept in water for 2 hr at the same temperatures. All sections were coated with L 4 emulsion. Radioautographs were developed after a 5 day exposure time.

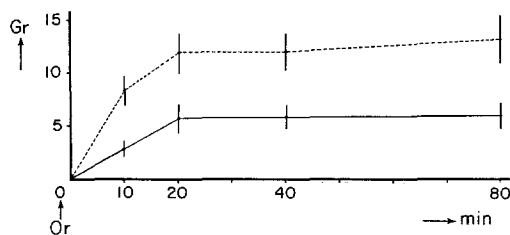


FIGURE 4 Grain counts in nucleus (solid line) and cytoplasm (broken line) of C₅₇Bl hepatic cells when labeling time is varied between 10 and 80 min. The vertical lines indicate the standard deviations.

RESULTS

Electron microscopy of hepatic tissue sections showed that, in general, the fine structure of the cells agreed well with detailed descriptions in the literature; but the fine structural appearance of the nuclear material requires discussion. Inside the nucleus a well defined, rather electron-scattering nucleolus, surrounded by a finely granular chromatin, is visible. This finely granular chromatin is continuous with the same type of material seen randomly dispersed in complexes throughout the nucleus (Fig. 3). This rather densely packed, finely granular chromatin is also found in a relatively small area along the inside of the nuclear envelope. On the strength of its localization and morphological appearance, this type of chromatin may be the equivalent of heterochromatin (11, 12). In addition, another more dispersed chromatin was found, scattered randomly throughout the nucleus

among areas of the condensed chromatin. This material occurs in an area sometimes referred to, in the literature, as the interchromatinic space (12). In our photographs of a cortisone-treated animal, it seems much more electron-opaque than the condensed chromatin. The material is granular or finely filamentous and contains aggregates of electron-opaque granules with a diameter of 250 to 450 Å (Fig. 3), designated in the literature as interchromatin or perichromatin granules (12) or nuclear ribosomes (13, 14).

In the high-resolution radioautographs, grains are found exclusively in the areas in which the dispersed chromatin occurs. No grains were found in areas containing condensed chromatin (Fig. 3). Grains were invariably found over the nucleolus. In the cytoplasm, grains are generally found in areas of ergastoplasm.

Grain counting was done light microscopically in radioautographs in 0.9 μ sections. As the photographs of these preparations show (Figs. 1 and 2), grains are present throughout the nucleus, but in the cytoplasm the grains are distinguishable from such cytoplasmic components as lysosomes, fat droplets, and mitochondria.

Fig. 4 shows the results of the experiments in which the labeling time was varied between 10 min and 80 min. The grain counts in nucleus and cytoplasm in the experiments with labeling times of 20, 40, and 80 min are remarkably constant. In the experiments with a labeling time of 10 min, the number of grains in both nucleus and cytoplasm is somewhat lower. In this series of experiments, we counted the grains in several tissue blocks from the same liver to test the reliability of the method. There were no significant differences between counts of two different blocks from the same liver.

The effect of hydrocortisone administration on the number of grains in the nucleus and cytoplasm was tested in three sets of experiments using rats and mice. The results are given in Table I. In all three experiments the grain counts in hydrocortisone-treated animals are significantly higher in both the nucleus and the cytoplasm.

Radioautographs of deparaffinized sections treated with RNase, unlike the control sections, did not reveal the presence of label.

DISCUSSION

From the data in the literature (1, 2) and the results of the present investigation, it is clear that grains in both light and electron microscope radio-

autographs represent the presence of labeled RNA molecules. With respect to the method of grain counting in radioautographs, statistical analysis of this material indicates that the reliability of the preparation and counting procedures applied is within acceptable limits. It may be assumed that the section thickness (0.9 μ), the use of a tritiated isotope, the exposure time, and the type and thickness of the emulsion chosen for the present study reduce to a minimum any errors that may result from such phenomena as cross-fire, image spread, self-absorption, and coincidence (15).

The results of the experiments in which the length of the labeling time was varied between 10 and 80 min show that in the initial 10 min the incorporation of the label is rather low, but rises to a higher, relatively stable level thereafter. This indicates that we are dealing with a relatively rapidly labeled RNA.

The experiments in which hydrocortisone was injected prior to labeling indicate that the hormone has a definite stimulative effect on the incorporation of the label: grain counts in both nucleus and cytoplasm were significantly increased. This may be explained in three ways, which have been discussed by Greenman et al. (16): (a) the rate of synthesis of the RNA is increased; (b) the half-life of the synthesized RNA molecules is lengthened; (c) cortisone may have had an influence on the precursor pool. These authors have reason to believe that the second and third possibilities can be ruled out. Our results can be correlated with Kidson and Kirby's findings (4) of a selective alteration of hepatic messenger RNA resulting from hydrocortisone treatment.

In an attempt to establish the localization of the label in the nucleus, we used high-resolution radioautography. Apart from a localization over the nucleolus, grains were found exclusively in nuclear areas that could be clearly distinguished from condensed chromatin. Such areas, containing several aggregates of granules, designated in the literature as perichromatin or interchromatin granules, probably correspond to the so called interchromatinic space described by Miyai and Steiner (12). Our findings with respect to the localization of labeled RNA are in agreement with those of Littau et al. (17), Karasaki (18), and Hsu (19).

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