

## DETECTION OF DNA BY TRITIATED ACTINOMYCIN D ON ULTRATHIN FROZEN SECTIONS

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### ABSTRACT

Ultrathin frozen sections of fresh liver tissue were floated on actinomycin D-<sup>3</sup>H. Quantitative high resolution radioautography was performed to determine the value of the method for detection of DNA by electron microscopy. A complete series of control experiments involving various treatments of frozen sections with enzymes (pronase, DNase) and 0.1 N HCl were also carried out to determine the specificity of the labeling. The results indicate the value of the method for detection of DNA directly on ultrathin frozen sections. Short treatments with pronase followed by DNase reduce the labeling to zero, whereas removal of chromosomal proteins with HCl increases the amount of radioactivity in the nucleus considerably. The results are discussed in view of the future applications opened by ultracryotomy, since radioautographic detection of various macromolecules and cellular components by labeled compound with specific affinities will now be possible.

### INTRODUCTION

Among the many possible applications of ultracryotomy, radioautographic studies are always mentioned first but, as of now, have failed to come through. Some of the reasons for this lie in the methodology of frozen sections: effectively, problems associated with freezing, sectioning, and handling the tissues reduce considerably the yield and preservation of frozen sections. Even more complex are the difficulties inherent in the radioautographic technique itself, especially when localization of soluble material is considered; fixation must be avoided and the material should never come in contact with any solvent (1, 11, 31).

There are, however, other radioautographic studies that could benefit from ultracryotomy. For example, the detection of macromolecules on frozen section by a "nonmetabolic" type of radioautography is possible. Labeled compounds with specific affinities for certain components could then be used as radioactive sources, provided the binding sites are preserved during the processing. Tritiated actinomycin D (AMD-<sup>3</sup>H) has been used in

this fashion at the light microscope level on squash preparations of fixed salivary glands (10) and oocytes (8, 9, 14, 15, 30).

Recently, we have developed a modified version of Bernhard's technique (6) for ultracryotomy based on the use of methyl cellulose as supporting medium which permits one to obtain large and numerous frozen sections with a commercial ultramicrotome (18). This paper presents a successful attempt to use AMD-<sup>3</sup>H for labeling of DNA directly on frozen sections. Several experiments on extraction and digestion were carried out and allow us to conclude that the binding is specific and similar to that reported on the basis of biochemical experiments (24).

### MATERIALS AND METHODS

#### *Ultracryotomy*

Normal adult rat liver was used for this study. Small pieces of fresh tissues (1 mm<sup>3</sup>) were fixed for 15 min and 1 hr at 4°C in 2.5% glutaraldehyde buffered

at pH 7.2 with 0.2 M sodium cacodylate. The tissues were washed overnight in the same buffer and soaked afterwards in a 25% glycerol solution for 10 min at room temperature (4). Processing for ultracryotomy was carried out with the help of methyl cellulose as supporting medium, followed by rapid freezing in liquid nitrogen. After trimming of the specimen, ultrathin frozen sections were cut with a Reichert OMU<sub>2</sub>-Fc 150 ultramicrotome with 50% dimethylsulfoxide (DMSO) as the flotation liquid. The cutting temperature was -50°C for the knife and -70°C for the specimen (18).

Since quantitative radioautography was contemplated, careful attention was given to choose sections of uniform thickness. Indeed, it should be remembered here that it is difficult to appreciate frozen section thickness, as the sections do not show color interference when floating on DMSO. With skill and practice, however, a regular ribbon of even sections can be obtained under optimal conditions when the microtome is performing regularly. Only such ribbon sections were collected with Marinuzzi rings (22), floated on decreasing concentrations of DMSO (40%, 30%, 10%), and stocked in distilled water in a watch glass until they were numerous enough for an experiment.

#### "Staining" with Actinomycin D-<sup>3</sup>H

AMD-<sup>3</sup>H at a specific activity of 8.4 Ci/mole was obtained from Schwarz Bio Research Inc. (Orangeburg, N. Y.). Watch glasses were previously washed overnight with an aqueous solution of cold AMD (Merck, Sharp & Dohme, 1 mg/ml) in order to saturate the surface since AMD binds strongly to glass. Incubation was carried out at 4°C by floating the sections from 1 min to 3 hr on a medium containing 100 μCi/ml of AMD-<sup>3</sup>H.

After incubation, the sections are transferred with a plastic ring (22) to a watch glass containing unlabeled AMD (1 mg/ml) for 1 hr at 4°C in order to remove, by isotopic exchange, labeled molecules that are not specifically bound to tissue. This step proved to be essential for reducing the nonspecific binding to tissue. When it is omitted, the labeling increases by 50% on both the nucleus and cytoplasm. Then the sections are floated over 20 successive baths of distilled water to remove unfixed AMD-<sup>3</sup>H, and deposited on glass slides previously coated with Formvar for radioautographic processing.

#### Control Experiments

Several sets of controls were designed in order to determine as clearly as possible the specificity of the labeling and the factors that could affect it. The following enzymatic solutions were used before AMD-<sup>3</sup>H labeling: (a) Deoxyribonuclease (DNase) 0.1% in

distilled water, pH 6.5, for 1 hr at 37°C, followed by two rinses in distilled water, (b) Pronase 0.1% in distilled water, pH 7.2, for 5 min, followed by human serum albumin, 1 mg/ml for 20 min, to remove enzymatic activity, and then three rinses in distilled water, (c) Pronase followed by DNase as described above, (d) Hydrochloric acid 0.1 N at 60°C for 1 hr, followed by two rinses in distilled water; (e) In one other set of controls DNase was used in (a) but after labeling with AMD-<sup>3</sup>H.

Another series of controls was intended to detect the possible influence of a postfixation on the stability of the binding. After labeling, the sections were floated on 2% osmium tetroxide followed by three rinses in distilled water. This series was, however, discarded because extensive damage to the tissue did not permit interpretation of the labeling.

In all of these control experiments the controls were assayed for two time periods of labeling with AMD-<sup>3</sup>H (15 min and 1 hr), but only the 1-hr labeling time period was considered for quantitative analysis.

#### Radioautography

The slides were coated by dipping into the Gevaert NUC 307 (Anvers, Belgium) emulsion diluted 1:3 with distilled water, and were exposed in the dark for 25 days at 4°C. Development was done according to the "gold intensification" procedure as modified with the use of an Elon ascorbic acid developer by Wisse and Tates (33). After fixation with buffered hyposulfite, the slides were dried, rinsed, and a 200-mesh grid was slid under the sections by the method of floating off the Formvar membrane (16).

The radioautographs were photographed unstained and without further treatment at 80kv with either a Philips EM 300 or a Siemens 101 electron microscope at a uniform magnification of 8000 with an objective aperture of 20 μ. An attempt was made to stain the sections by acid hydrolysis of the gelatin (H<sub>2</sub>O distilled, 37°C, 30 min; 0.5 N acetic acid, 37°C, 15 min) followed by 0.5% uranyl acetate, 30 min, and lead citrate, 1 min (6). However, from our experience with labeling, such treatment reduced considerably the number of grains and hence was abandoned.

For all series, the same method of quantitation was used, the concentration of the radioactivity over the nucleus and adjacent cytoplasm was estimated by counting the grains on the photographic plates, and the surface was approximated by superposition of a transparent sheet calibrated in square microns. For each series, the background was calculated by sampling four or five areas on the grids outside the sections and by deducing them from the total grain count. This background never exceeded 0.04 grain per square micron, and was identical before and after exposure. Therefore, positive chemography (27) was considered to be absent. Negative chemography due

to the technique and chemicals used for cryomicrotomy was measured by comparing radioautographs of tissue processed through the classical double fixation-Epon embedding technique with radioautographs processed through cryomicrotomy; no significant difference was noted. The system used for this experiment was thymidine- $^3\text{H}$ -labeled hamster cells in tissue culture.

## RESULTS

### *General Observations*

The introduction of methyl cellulose as supporting medium for ultracryotomy allows easy and regular sectioning and facilitates quantitative radioautographic analysis. After 1 hr of fixation with glutaraldehyde and staining with 0.5% uranyl acetate for 10 min and lead citrate for 1 min, liver cells are well preserved and all nuclear components are readily recognizable including the nucleolus, nuclear pores, chromatin, and peri- and interchromatin granules. The cytoplasm is intact with all membranes negatively stained. Mitochondrial matrix and cristae as well as rough endoplasmic reticulum can be easily identified (Fig. 1). Glycogen appears as blank areas, but its preservation can be demonstrated by phosphotungstic acid at pH 1 (18). Tissues fixed for 15 min only in glutaraldehyde showed a poorly preserved ultrastructure and therefore were not used for quantitative analysis.

The necessary use of a flotation liquid, numerous rinses, and enzymatic treatments did not alter the preservation as will be seen below. Cryoprotection with glycerol does not appear to be a necessary step but seems to ameliorate ultrastructural preservation. All radioautographs were photographed unstained without removal of the gelatin, and the contrast was sufficient to permit ultrastructural and quantitative analysis.

### *"Staining" of Floating Sections with Actinomycin D- $^3\text{H}$*

When sections are floated on AMD- $^3\text{H}$  for 1 hr, most of the radioactivity is located over the nucleus, with little activity in the cytoplasm. There appears to be no particular preferential localization within nuclear compartments such as nucleolus, diffused or condensed chromatin. Some of the cytoplasmic silver grains were found around the nucleus and probably arise from particles emitted by a tritium source in the nucleus. Mitochondria

were labeled occasionally, but no attempt was made to prove the specificity of this radioactivity (Fig. 2).

The action of pronase at 0.1% even followed by serum albumin reduced the amount of labeling, but at this concentration it has been shown that deoxyribonucleoproteins are affected (23). On the other hand, DNase used either before or after the "staining" with AMD- $^3\text{H}$  does not significantly affect the amount of activity. These results do not come as a surprise, as DNase has no effect on tissues fixed with aldehydes if not preceded by treatment with a proteolytic enzyme (22). In Fig. 3, the section was treated with pronase followed by DNase before labeling with AMD- $^3\text{H}$  for 1 hr. Compared with Fig. 2, the radioactivity has completely disappeared from the section. The preservation of the sections remains quite good even after the combined action of these enzymes.

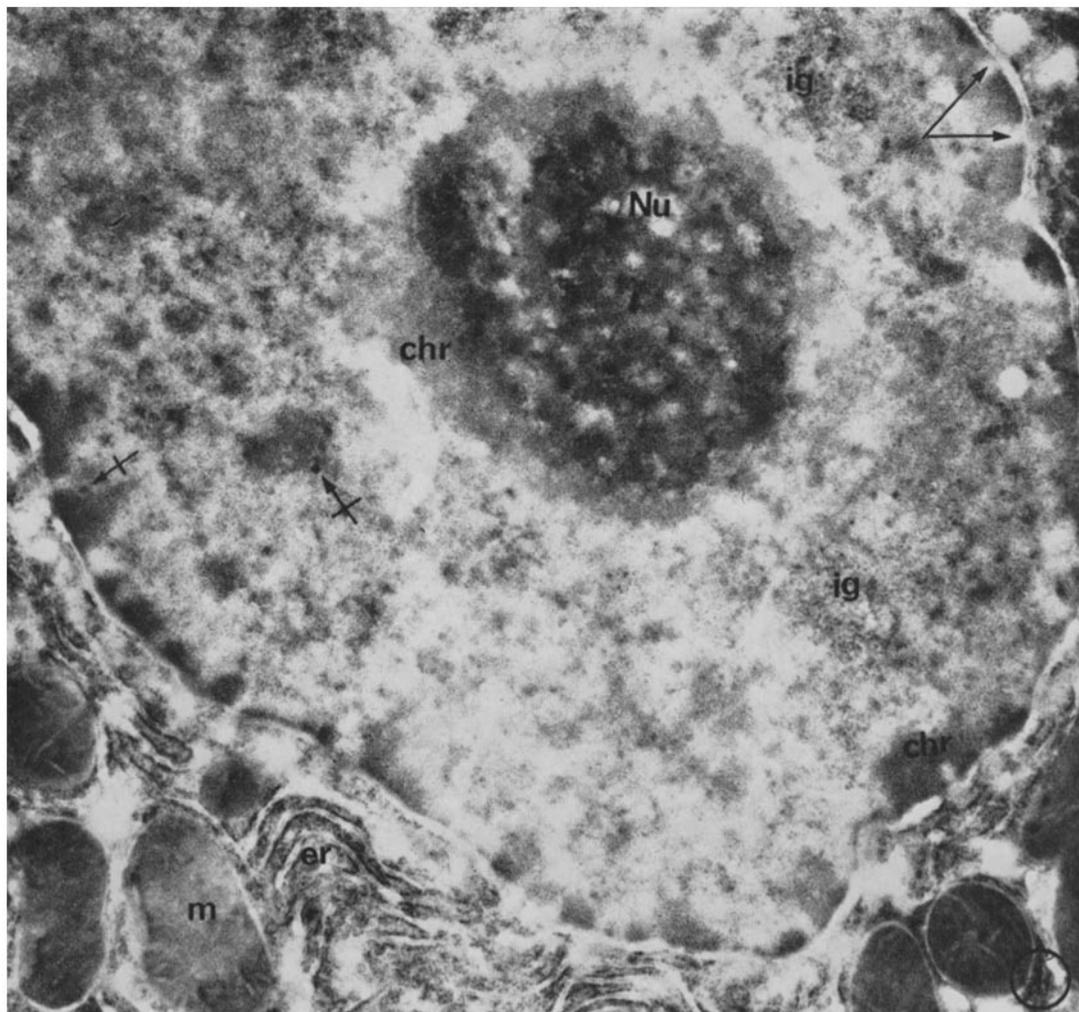
In another set of experiments, labeling for 1 hr with AMD- $^3\text{H}$  was preceded by a treatment with 0.1 N HCl for 1 hr, a treatment which is known to remove the chromosomal proteins (2, 19). Such a treatment resulted in a spectacular labeling of the nuclei (Figs. 5 and 6) as compared with the control experiments in which no pretreatment with HCl was done (Fig. 4).

Comparison of hepatocytes and reticuloendothelial cells showed that in all experiments the Kupffer cells were more heavily labeled than any other cell (Fig. 7).

### *Quantitative Analysis*

Table I gives a quantitative analysis of the amount of labeling as a function of time of flotation on AMD- $^3\text{H}$ . The number of silver grains is reported on the surface in square microns and each nucleus has been compared with its adjacent cytoplasm. The statistical significance test "F" (variance analysis), standard deviation, and probability were calculated for each time of treatment. The results indicate a substantial increase in radioactivity as a function of time for the nucleus, whereas this increase is negligible in the cytoplasm for a short time of treatment. By 15 min of "staining" with the labeled antibiotic, the concentration of the radioactivity in the nucleus is significantly elevated over that of the cytoplasm.

The labeling time of 1 hr was chosen for the control experiments with enzymatic extractions. Table II and Fig. 8 show the comparative results for the nucleus and the cytoplasm. Pretreatment with pro-



All the tissues presented here were fixed for 1 hr in glutaraldehyde, soaked in 25% glycerol for 10 min, embedded with 3% methyl cellulose, and frozen in liquid nitrogen. All sections were made with the Reichert cryo-ultramicrotome.

**FIGURE 1** Portion of rat liver cell stained with 0.5% uranyl acetate for 10 min and lead citrate for 1 min showing the over-all preservation of ultrathin frozen section used in this study. The nuclear components of the rat liver cell are readily recognizable: nucleolus (*Nu*), nuclear pores (→), chromatin (*chr*), peri- (†) and inter- (*ig*) chromatin granules. In the cytoplasm, mitochondria (*m*) with cristae are easily identified while ergastoplasm (*er*) stands out. Membranes are negatively stained.  $\times 30,000$ .

nase followed by DNase reduces the radioactivity over the nucleus and cytoplasm almost to zero. Pronase alone also causes reduction of the number of silver grains, whereas DNase has little or no effect. However, the labeling of nuclei after treatment with HCl increases threefold, with little variation in the cytoplasm.

#### DISCUSSION

One of the major goals in the use of ultrathin frozen sections, as stated by Bernhard and Nancy (4) and Bernhard and Leduc (5), is to preserve the biochemical reactivity and the fine structure of cellular components. Several applications have already been made; to mention a few: cytochemical

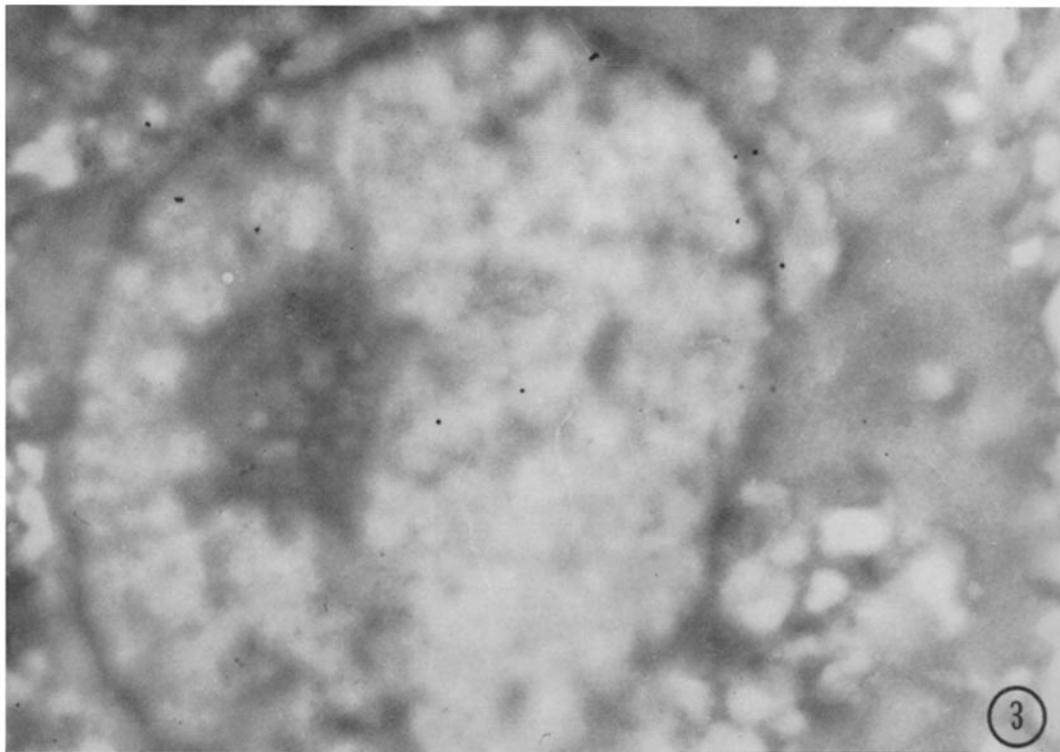
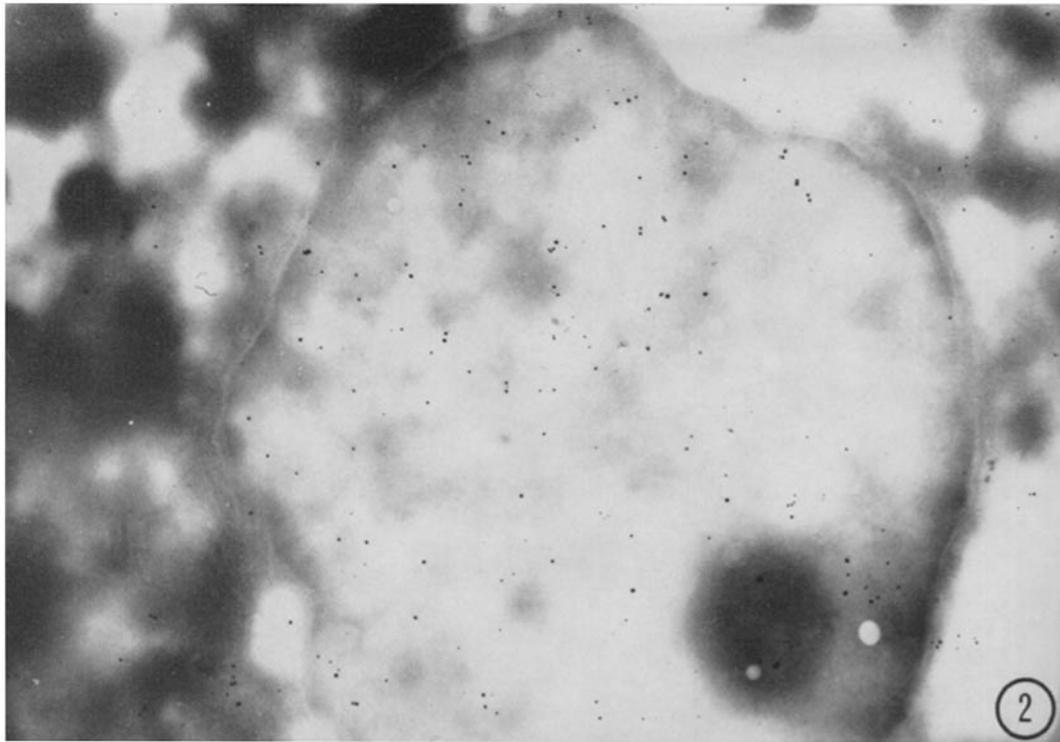


FIGURE 2 Radioautograph of an unstained section of rat liver cell floated on AMD-<sup>3</sup>H for 1 hr. Note the heavy labeling of the nucleus as compared with its cytoplasm.  $\times 15,300$ .

FIGURE 3 Radioautograph showing a cell from the pronase-DNase pretreated series that was incubated with AMD-<sup>3</sup>H for 1 hr. Compared with Fig. 2, only a few grains are seen in the nucleus while the cytoplasm has no activity. Unstained.  $\times 15,300$ .

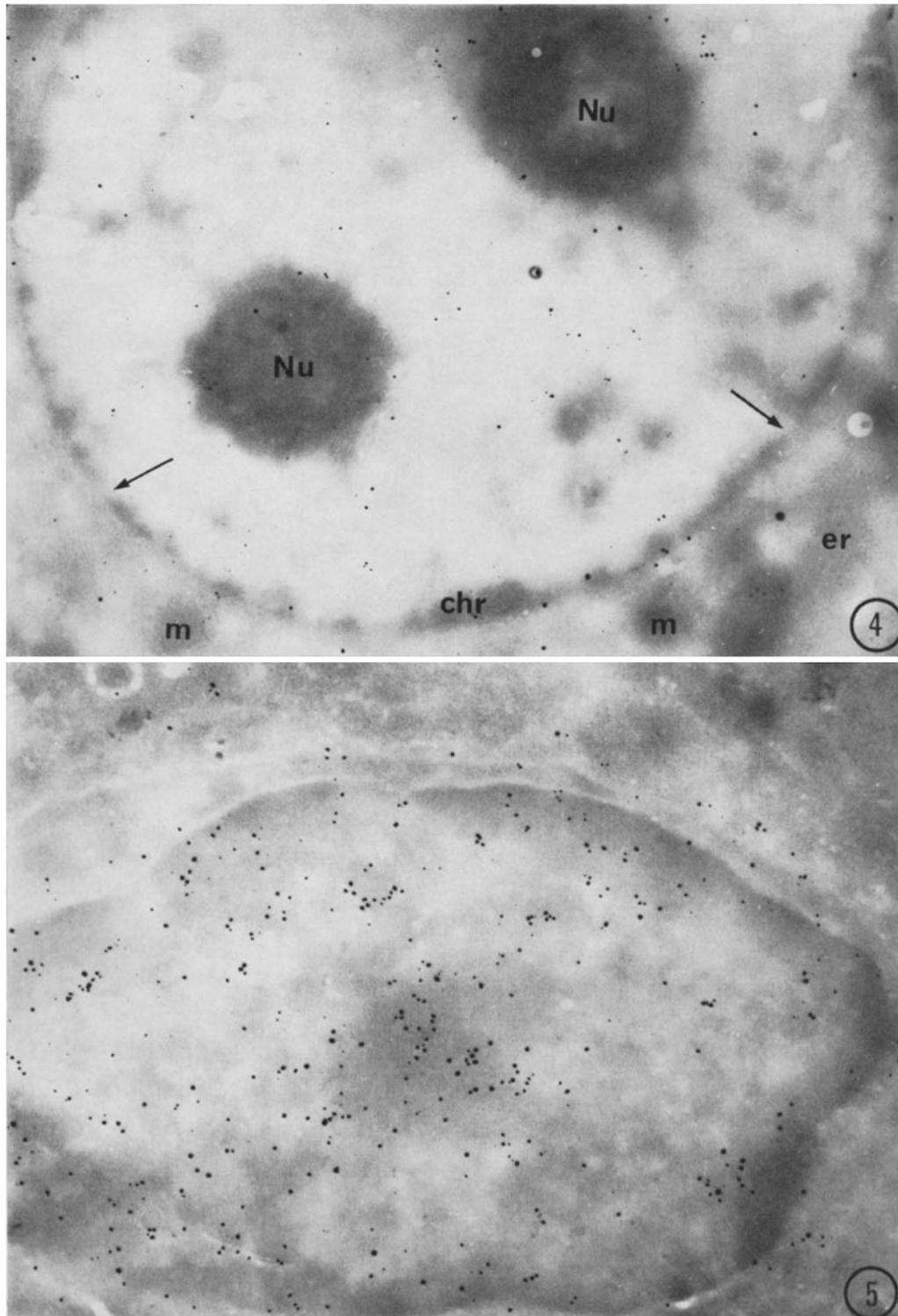


FIGURE 4 Sections floated on AMD-<sup>3</sup>H for 1 hr with the same distribution of grains as in Fig. 2. Note here the ultrastructural aspects of unstained, glutaraldehyde-fixed tissue. There was no difficulty for identification of the nucleus from the cytoplasm, nucleolus (*Nu*), chromatin (*chr*), nuclear pores (→), and even the rough endoplasmic reticulum (*er*) and mitochondria (*m*). Unstained.  $\times 16,000$ .

FIGURE 5 This section was pretreated with 0.1 N HCl for 10 min, washed, and floated on AMD-<sup>3</sup>H for 1 hr. A striking increase in labeling is evident in the nucleus. Compare with Fig. 4. Unstained.  $\times 14,000$ .

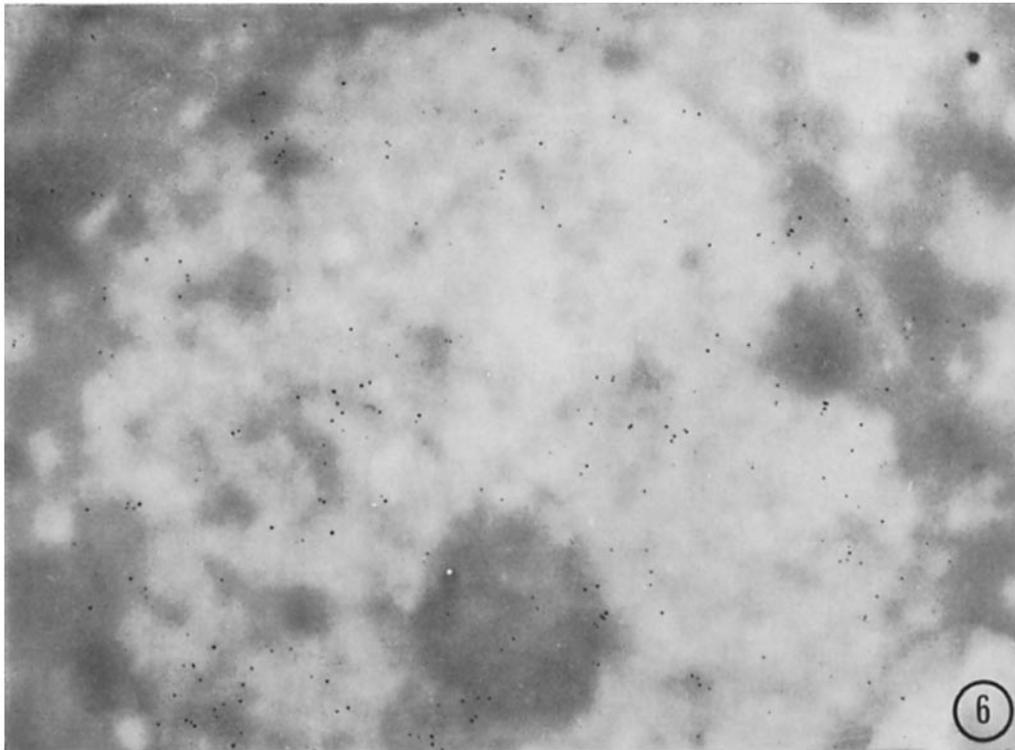


FIGURE 6 Another cell from the HCl pretreated series showing the heavy labeling of the nucleus as compared with Fig. 4. A quantitative analysis of the intranuclear distribution of this series did not reveal a preferential distribution of labeling over any particular nuclear structure. Unstained.  $\times 15,000$ .

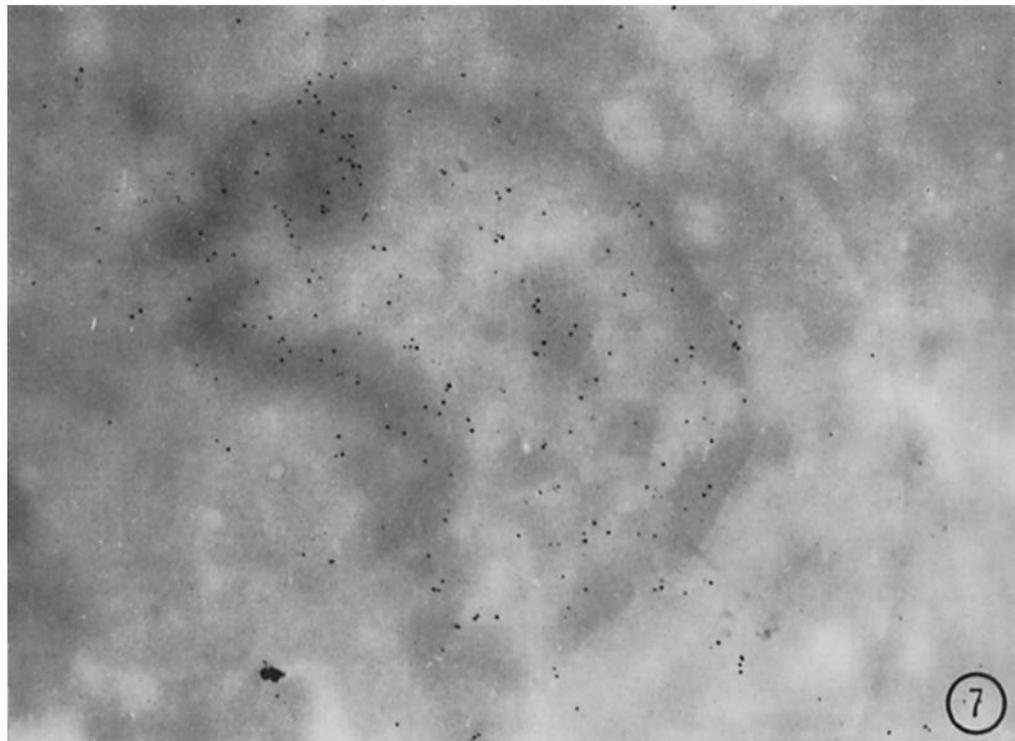


FIGURE 7 Kupffer cell from a section floated for 1 hr on AMD- $^3\text{H}$ , without any other treatment. Note the important labeling over the nucleus and compare with Figs. 2 and 4. The labeling over such nuclei appears nearly as heavy as that over the nuclei of the HCl pretreated series. Unstained.  $\times 20,000$ .

TABLE I  
Distribution of the Radioactivity as a Function of Time in Frozen Ultrathin Sections of Liver Cells Floated on AMD-<sup>3</sup>H

	No cells	$\bar{x}/N$	$\sigma N$	$\bar{x}/C$	$\sigma C$	GC	F N/C	P
<i>min</i>								
1	11	1.36	±1.42	0.37	±0.20	1017	5.17	>5/10 <sup>2</sup>
5	19	1.48	±0.60	0.36	±0.41	1639	44.45	>1/10 <sup>3</sup>
15	21	3.85	±1.62	0.67	±0.33	2246	77.95	>1/10 <sup>3</sup>
60	17	3.89	±3.08	0.75	±0.55	1703	17.13	>1/10 <sup>3</sup>
180	18	6.43	±1.33	2.18	±0.66	3864	147.69	>1/10 <sup>3</sup>

$\bar{x}$ , mean grain counts per square micron over the nucleus ( $\bar{x}/N$ ) or cytoplasm ( $\bar{x}/C$ ).  $\sigma$ , standard deviation for each time of treatment in the nucleus ( $\sigma N$ ) and cytoplasm ( $\sigma C$ ). F, statistical significance (variance analysis). P, probability. GC, total grain count.

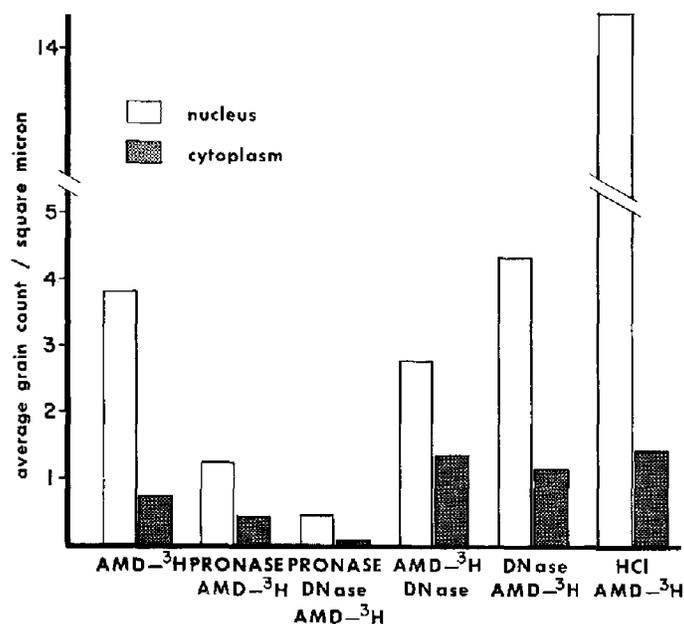


FIGURE 8 Comparative histogram of the control series for the same labeling of 1 hr with AMD-<sup>3</sup>H. The grain counts over the nucleus and cytoplasm were classified as exclusive and attributed to only one structure. Enzymatic treatments were used as described in Materials and Methods.

demonstration of endogenous enzymes (20, 32, 34) and complex carbohydrates (18), and localization of enzyme-labeled antibodies (21). Modification of the technique should allow radioautographic detection of soluble and diffusible substances at the electron microscope level.<sup>1</sup> This paper demon-

<sup>1</sup> Iglesias, R., R. Bernier, and R. Simard. 1972. Localization of soluble iodide in the thyroid gland using ultrathin frozen sections. In preparation.

strates that ultracytometry will permit other radioautographic applications that were regarded, up to now, as highly hypothetical, as far as electron microscopy is concerned.

The specificity of AMD-<sup>3</sup>H binding to DNA on frozen sections is evident by qualitative and quantitative analysis of the radioautographs. Indeed, the radioactivity is high in the nuclei where DNA is known to exist, and low in the cytoplasm. Cyto-

TABLE II  
Comparative Results of Control Experiments for the Same "Staining" Time of AMD-<sup>3</sup>H of 1 hr

	No cells	$\bar{x}/N$	$\sigma_N$	$\bar{x}/G$	$\sigma_G$	GC	FN/C	P
AMD- <sup>3</sup> H (60 min)	17	3.89	±3.08	0.75	±0.55	1703	17.13	>1/10 <sup>3</sup>
Pronase-AMD- <sup>3</sup> H	18	1.20	±0.44	0.27	±0.15	614	84.02	>1/10 <sup>3</sup>
Pronase DNase AMD- <sup>3</sup> H	23	0.34	±0.23	0.05	±0.00	210	8.10	>1/10 <sup>3</sup>
DNase AMD- <sup>3</sup> H	21	4.32	±1.17	1.12	±0.26	2570	149.59	>1/10 <sup>3</sup>
AMD- <sup>3</sup> H DNase	18	2.76	±1.23	1.36	±0.61	2950	18.79	>1/10 <sup>3</sup>
HCl AMD- <sup>3</sup> H	19	14.65	±10.66	1.47	±0.69	5018	28.86	>1/10 <sup>3</sup>

Pronase was used at 0.1% for 5 min, pH 7.2, DNase 0.1%, pH 6.5, 1 hr; HCl, 0.1 N at 60°C for 1 hr. Same symbols as in Table I.

plasmic labeling in hepatocytes is greater than background level, and also is greater than the labeling seen over structures known not to contain DNA (red blood cells, blood vessels, fibrous tissue etc.). Part of this labeling appears to be associated with mitochondria, but no quantitative study was carried out. Furthermore, there is a total absence of labeling when the action of DNase is made possible by a short pretreatment with pronase. Enhancement of the labeling is obtained by treatment with HCl at low concentration (0.1 N). This result is consistent with the fact that removal of chromosomal proteins results in an increase in the AMD-<sup>3</sup>H binding sites on the DNA (2, 7). More concentrated HCl solutions (1 N) have been used to obtain a reverse effect, i.e. suppression of labeling through depurination of DNA in squash preparations of salivary glands (10).

There are other examples of the use of AMD-<sup>3</sup>H as a cytochemical label for radioautographic detection of DNA at the level of the light microscope (8, 9, 10, 14) as well as the electron microscope (29, 30), but attempts to use floating sections have failed so far<sup>2</sup> (30) because of the high and nonspecific background level, which is probably due to the embedding medium, whether Epon or glycol methacrylate. Such background was not observed in our experience, and in fact the only background observed was that of the Gevaert emulsion. The advantages of "staining" floating sections on AMD-<sup>3</sup>H or other labeled molecule is obvious, as the method eliminates the problems of penetration of

fixed or unfixed tissues and could permit the detection of small amounts of biological molecules.

The incorporation of labeled AMD-<sup>3</sup>H in vivo was shown to be a function of the time of treatment and resulted in a heavy labeling of the condensed heterochromatin (3, 28, 29, 30). This preferential localization was not observed in the present study, but there was a definite increase in the radioactivity as a function of time of treatment. These results have been observed by others in ascites tumor cells (17) and lymphocytes (25). According to Ringertz et al. (26), there are at least two types of binding, an early and rapid form, and a second, slow form. Our results are consistent with this observation. Early treatments with AMD-<sup>3</sup>H resulted in a rapid labeling until 15 min, afterwards, the silver grains increased much more slowly. Darzynkiewicz and Anderson (12), Darzynkiewicz and Jacobson (13), and Steinert and Van Gansen (30) observed that cells with small and condensed nuclei are labeled much more heavily than any others. A similar phenomenon was noticed in the present study by the heavy labeling of Kupffer cells which have small and condensed nuclei.

In conclusion, a new procedure for the detection of DNA by floating ultrathin frozen sections on AMD-<sup>3</sup>H has been developed for electron microscopy. The specificity is high and the method may well become very useful for the detection of cellular components by drugs with specific affinities, since the problem of penetration is eliminated. This approach can also be used for quantitative analysis, provided a good series of controls is carried out concomitantly.

<sup>2</sup> Simard, R. 1967. Unpublished results.

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