A METHOD FOR INTRACELLULAR AUTORADIOGRAPHY IN THE ELECTRON MICROSCOPE

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ABSTRACT

A technic is described for high resolution intracellular autoradiography in the electron microscope. Cultures of LLC-MK$_2$ monkey kidney cells were incubated for 72 hours in a medium containing 0.4 μcurie per ml of thymidine-H^3. After labeling, the cells were fixed with osmium tetroxide and embedded in methacrylate. Ultrathin sections of the labeled tissue were taken up on Formvar-coated and carbon-stabilized electron microscope grids. A 150 to 450 A layer of silver metal was then evaporated onto the tissue. The coated grids were exposed to bromine vapor for 1.5 to 2 minutes under red light, allowed to dry for 1 minute, and then covered with a thin film of 1 per cent aqueous gelatin applied by means of a fine wire loop lowered over the grid supported on a glass peg. For autoradiographic exposure, the grids were stored 50 days in a light-proof container at 4°C with calcium chloride desiccant. Development was carried out for 5 minutes at 20°C in Promicrol (May and Baker, England) diluted 1:1 with water, followed by a 1 minute water wash and fixation for 2.5 minutes in 15 per cent aqueous sodium thiosulphate. After removal of the gelatin by immersion for 16 hours in water at 37°C, the autoradiograms were dried and examined in the electron microscope. Ultrastructural detail was fairly well defined and the cytoplasm of each labeled cell was covered with an electron opaque deposit of silver, suggesting that a polynucleotide containing thymidine may be synthesized in the cytoplasm. The matter is discussed.

INTRODUCTION

Recent advances in the understanding of cell ultrastructure, together with new findings concerning the biochemistry of cell particulates, have made it imperative that a means should now be found for localization of enzyme-active centers and other sites in accurate relation to ultrastructural detail. Autoradiography has been applied to this end with considerable success, but only at magnifications obtainable in an optical microscope.

The first attempt to photograph autoradiograms in the electron microscope was made by Liquier-Milward (1), who placed radioactive cell nuclei on a Formvar film and covered them with a thin layer of Ilford G5 emulsion. After exposure and development, the autoradiograms were examined at a magnification of approximately 6000. Tracks of beta rays emanating from dense black silver conglomerates were observed against the diffuse mottled background of emulsion gelatin, but the outlines of the cell nuclei were not discernible.

Subsequent to this attempt, O'Brien and

George (2) prepared autoradiograms using a thin layer of Eastman Kodak NTA emulsion to cover ultrathin sections of yeast cells which had been incubated with polonium-210. Electron micrographs of these autoradiograms showed the vague outlines of the yeast cells with dense alpha particle tracks, and also dark spots indicating the location of the isotope at the cell periphery.

In a later experiment George and Vogt (3) placed radioactive dust particles on electron microscope grids covered with a thin layer of Eastman Kodak NTA emulsion. After exposure and development, these autoradiograms revealed dense ionization tracks emanating from the radioactive source, but the outlines of the original dust particles were obscured by a heavy overlying deposit of silver.

For accurate localization of a radioactive source within a cell or otherwise, it is desirable that particle tracks should be avoided by making the overlying emulsion as thin as possible. With an ideally thin layer, very few ionizing particles will cause tracks in the plane of the emulsion. The remainder will escape beyond the emulsion, ionizing only the silver halide particles immediately overlying their source. The autoradiograms will therefore consist of a series of discrete silver granules which can be accurately related to cell ultrastructure.

A thick layer of overlying photographic emulsion has the additional disadvantage that it obscures ultrastructural detail in the electron microscope.

An autoradiographic technic has therefore been devised to permit removal of the gelatin layer before examination in the electron microscope. Ultrathin sections of radioactively labeled tissue are covered with a thin film of evaporated silver metal. This is exposed to bromine vapor, thereby forming a fine deposit of silver bromide firmly attached to the tissue by powerful Van der Waals cohesive forces. The section is covered with a thin layer of gelatin to prevent autodevelopment of the silver halide, and is then stored for several weeks at low temperature in a light-proof container with desiccant. After exposure, the autoradiograms are developed and fixed. The gelatin layer is washed away with water, leaving the developed silver granules firmly attached to the tissue at the locations of the radio-isotope. These autoradiograms show ultrastructural detail in the electron microscope since there is no overlying gelatin to interfere with the electron beam.

MATERIALS AND METHODS

LLC-MK₂ Monkey Kidney Cells

The strain was isolated from cells obtained by trypsinization of six pairs of rhesus monkey kidneys on March 9, 1955 (4). A culture in the 120th passage was obtained from Dr. Robert N. Hull of Lilly Research Laboratories on June 29, 1959, and has since been subcultured at the South African Poliomyelitis Research Foundation in Parker 199 medium containing 5 per cent of horse serum. The average intermitotic interval for the LLC-MK₂ strain is 37 hours, with a median time of 34 hours and a range from 25 to 64 hours (5). The cells are conveniently large (20 to 30 μ diameter) for autoradiographic studies, but are often somewhat granular in appearance under the optical microscope.

Incubation of LLC-MK₂ Strain Cells with Thymidine-H^3

A freshly trypsinized subculture of approximately 4 million LLC-MK₂ cells was added to 10 ml of Parker 199 nutrient medium containing 5 per cent of horse serum. To the suspension was added 0.1 ml (4 μcuries) aqueous thymidine-H^3 (obtained from the Radiochemical Centre, Amersham, England as thymidine-(nominally-6-T) in distilled water with a specific activity of 4.4 curies per millimole). The effective specific activity of the thymidine-H^3 in the final suspension was probably lowered owing to the presence of 0.3 μg per ml of unlabeled thymine in the Parker 199 component. A final concentration of 0.4 μcurie per ml thymidine-H^3 is safely below the radiation toxic level reported by Painter, Drew, and Hughes (6) for HeLa cells, and does not affect the growth rate of L strain mouse cells (7).

Culture tubes were inoculated with 1 ml each of the suspension in radioactive medium, and allowed to stand 48 hours at 37°C before being placed on a roller wheel. After 72 hours growth the tubes were emptied, washed 10 times with Hank's solution, and replaced for 5 hours in the incubator at 37°C after refilling with 1 ml of Parker 199 medium containing 5 per cent of horse serum but no isotope. This was to permit leaching out of any thymidine-H^3 not incorporated into polynucleotide or otherwise bound within the cells.

Fixation and Embedding of Radioactively Labeled Cultures

After incubation with isotope, the cells were fixed and embedded in one of two ways:

a) The culture tube was emptied and the medium replaced by 1 ml of Earle's BS solution containing 0.25 per cent w/v of trypsin. The cells became

detached from the tube wall after 20 to 30 minutes incubation at 37°C, and were centrifuged down from the medium at approximately 50 g for 5 minutes. Fixation was carried out according to Palade (8) by resuspending the cells for 30 minutes at 0°C in veronal-acetate buffer (pH 7.4) containing 1 per cent osmium tetroxide. After dehydration in graded alcohols the cell mass was embedded in a mixture of methyl and butyl methacrylates (1:9) polymerized with 2 per cent Luperco CDB at 56°C.

b) The culture tube was emptied and placed on a gently inclined rack with the cell sheet uppermost. Fixation was carried out for 20 minutes in the vapors of 2 per cent aqueous osmium tetroxide introduced beneath the cells, which were thereafter dehydrated in graded alcohols and embedded directly in methacrylate according to the method of Hotchin et al. (9). The embedded cells were separated from the glass by cracking away the tube in dry ice.

Preparation of Autoradiograms

Ultrathin sections of the radioactively labeled cells were prepared on a Servall Porter-Blum ultra-microtome fitted with a diamond knife obtained from the Venezuelan Institute for Scientific Investigation, Caracas.

The sections were caught up on Formvar-coated electron microscope grids which had been lightly stabilized with carbon. In earlier experiments the Formvar film and tissue frequently detached from the grid during development of autoradiograms. To prevent this the grid was first rendered "tacky" by dipping into a solution of 0.5 per cent Formvar in chloroform and drying immediately on filter paper, thereby encasing the meshes in a coat of Formvar but leaving the pores open. Formvar films adhered satisfactorily to such precoated grids.

For silvering, the grids were placed horizontally with adhering tissue sections face downward, 8 inches above a spiral tungsten wire inside the dome of a vacuum coating plant (W. Edwards and Co., London, model 12E/154). Small strips of pure silver foil (160 mg total) were draped over the tungsten wire and the apparatus was evacuated to 10^{-4} mm mercury. Electrical heating of the tungsten spiral caused evaporation of the silver to produce a bright mirror over the tissue section and Formvar support.

The silvered grids were then placed on top of glass pegs (3.5 mm in diameter and 3 cm high) supported in an upright position around the circum-ference of a Perspex base (5 mm high and 25 mm in diameter) fitted with a central rod (3.5 mm in diameter and 65 mm high) to serve as a handle.

Under a red darkroom light (Agfa Schutz filter L.107) the "cruet" of grids was lowered into a wide mouthed 2 ounce bottle containing a few drops of liquid bromine. After 1.5 to 2 minutes exposure to bromine vapor, the grids were withdrawn with a white coating of silver bromide in place of the bright silver metal.

Each grid was then covered with a 1 per cent aqueous solution of gelatin, applied as a thin film by means of a flat wire loop 4 mm in diameter lowered over the grid supported on its glass peg. After drying over calcium chloride in a light-tight vessel, the grids were removed from their glass pegs and placed on filter paper in Petri dishes containing small calcium chloride desiccators. The Petri dishes were then stacked in light-proof metal cans with additional desiccant. These were sealed with surgical tape and stored at 4°C for autoradiographic exposure.

Development and Examination of Autoradiograms

After 50 days exposure the grids were removed from their containers under red light. Each grid was grasped at its edge with jeweler's forceps held clamped by two small clothes pegs set at right angles to the blades of the forceps. The grid was then lowered into a 50 ml beaker of Promicrol developer (May and Baker, England) diluted 1:1 with distilled water, and supported by resting the clothes pegs against the rim of the vessel. Development was carried out for 5 minutes at 20°C. The grid was then washed 1 minute by immersion in distilled water, and fixed for 2.5 minutes in 15 per cent aqueous sodium thiosulphate. After a further 2 minute water wash, the grid was immersed for 16 hours in a Petri dish of water at 37°C to remove the gelatin, and then dried in a calcium chloride desiccator. The autoradiograms were examined in either a Metropolitan-Vickers model E.M. 3 or a Siemens Elmiskop I electron microscope.

RESULTS

Preliminary experiments showed that the 150 to 450 A particles of the evaporated silver mirror (Fig. 1) condensed to larger 250 to 1100 A particles of silver bromide (Fig. 2) on exposure to bromine vapor. The coverage was uniform and adequate to provide good autoradiographic resolution over cells 20 to 30 μ in diameter.

Fixed and embedded LLC-MK₂ cells which had been incubated with thymidine-H³ were checked for radioactivity in an EKCO scintillation counter, model 664 A. A shaving of plastic-embedded cells (16 mm square) was placed in a counting jar with a solution of 0.3 per cent diphenyl oxazole scintillator in toluene. The plastic dissolved away leaving the cells in suspension. A similar quantity of pure plastic without cells was added to the
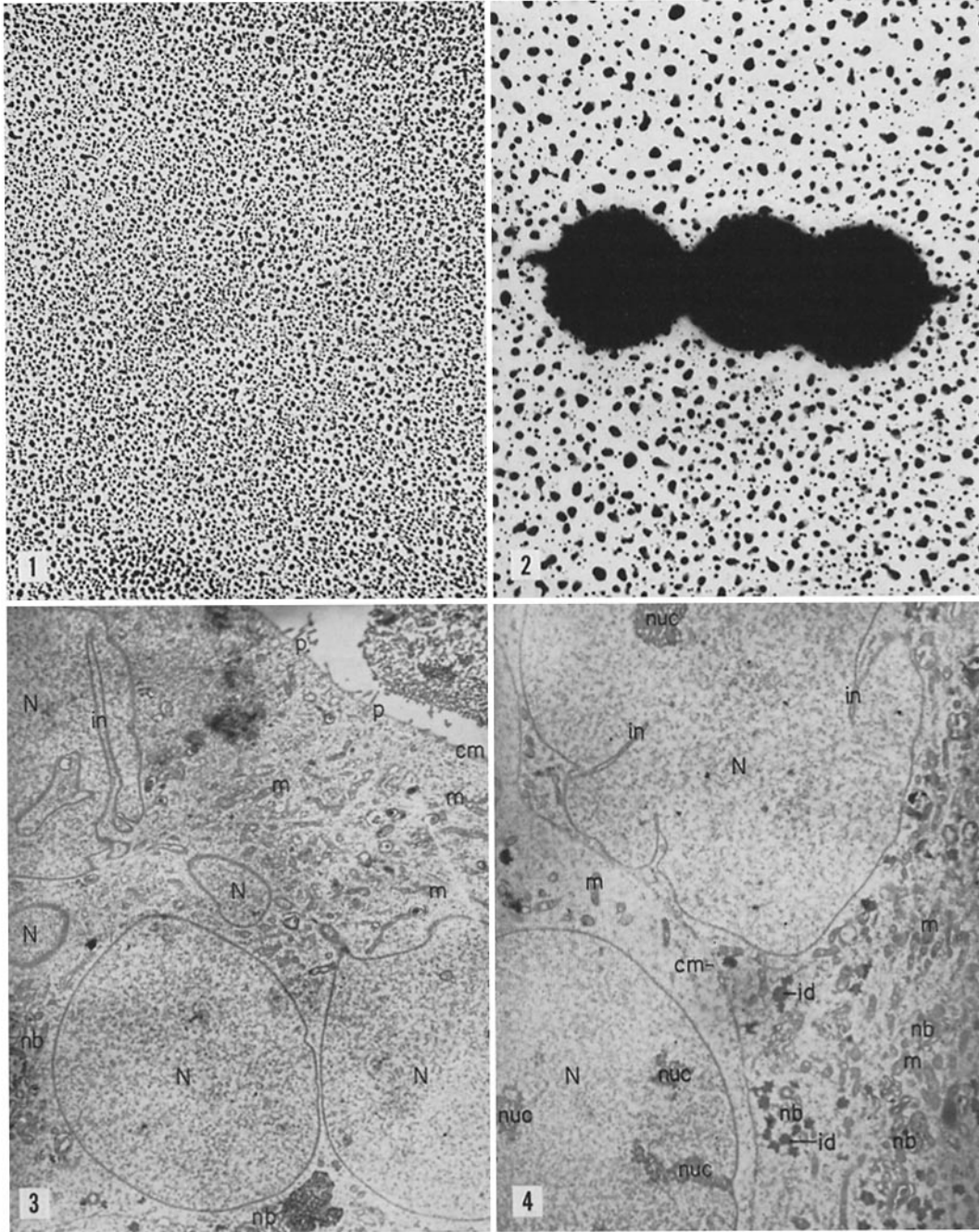
blank control. The test suspension showed a count of 10 times the control background, proving that the cells were radioactively labeled.

Figs. 3 to 6 show "control" LLC-MK₂ cells prior to coating with the evaporated photographic emulsion. These tissue culture cells sometimes have as many as four to six nuclei, which are often deeply invaginated and distorted. Mitochondrial ultrastructure was well preserved in the specimens studied, but the endoplasmic reticulum was not well defined. This may represent a loss of ultrastructural detail during tissue culture, or may be a result of the embedding technic used.

Autoradiograms of LLC-MK₂ cells incubated with thymidine-H³ are shown in Figs. 7 to 15. A dense deposit of metallic silver is seen overlying the cytoplasm, with virtually none in the nucleus or outside the cell. Of the cells studied, approximately 60 per cent were labeled in the cytoplasm, while the remainder were unlabeled. No nuclear labeling was observed except in the single instance of an isolated nucleus stripped of its cytoplasm.

DISCUSSION

The results show very clearly that intracellular autoradiography in the electron microscope is now a practical possibility. A method has been devised to permit examination of the autoradiogram without an overlying film of gelatin which might otherwise obscure ultrastructural detail. In previous methods involving the use of stripping film, removal of the gelatin emulsion would also remove the developed silver particles suspended in it. Photographically sensitive silver halide particles formed in direct contact with the radioactively labeled tissue will be held in position by powerful cohesive forces and will not shift during development and processing of the autoradiograms. Artifacts such as would arise from the shifting of a stripping film relative to the tissue are thus obviated.

The method has the additional advantage that confusing ionization tracks are unlikely to be registered, since only those radiations traveling in the plane of the silver halide layer (approximately

Key to Labeling

N, nucleus	*id*, inclusion droplet
nuc, nucleolus	*in*, invagination of nucleus
m, mitochondria	*p*, pseudopodia
cm, cell membrane	*nb*, "nebenkern" structures

Figure 1

Metallic silver evaporated onto a Formvar supporting film. Radioactive tissue sections were covered with a similar deposit as an initial step in the preparation of autoradiograms. Particle size 150 to 450 A. × 15,000.

Figure 2

Silver bromide particles on a Formvar film. The metallic silver deposit of Fig. 1 was exposed 1.5 to 2 minutes to bromine vapor. Dark spheres are polystyrene-latex (1.170 μ in diameter, also exposed to bromine). Particle size 250 to 1100 A. × 17,000.

Figure 3

Portion of a polynucleated LLC-MK₂ rhesus monkey kidney cell prior to coating with photographically sensitive silver bromide particles. One nucleus (*N*) is deeply invaginated (*in*), and numerous mitochondria (*m*) are to be seen in the cytoplasm. × 3,500.

Figure 4

Two adjacent LLC-MK₂ rhesus monkey kidney cells prior to coating with photographically sensitive silver bromide particles. One of the two nuclei (*N*) is deeply invaginated (*in*), and both have nucleoli (*nuc*). Numerous mitochondria (*m*) and "nebenkern" structures (*nb*) may be seen in the cytoplasm. × 3,500.
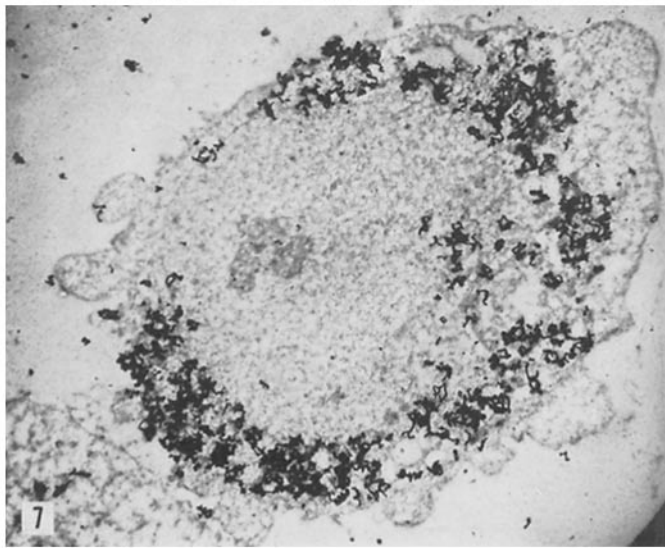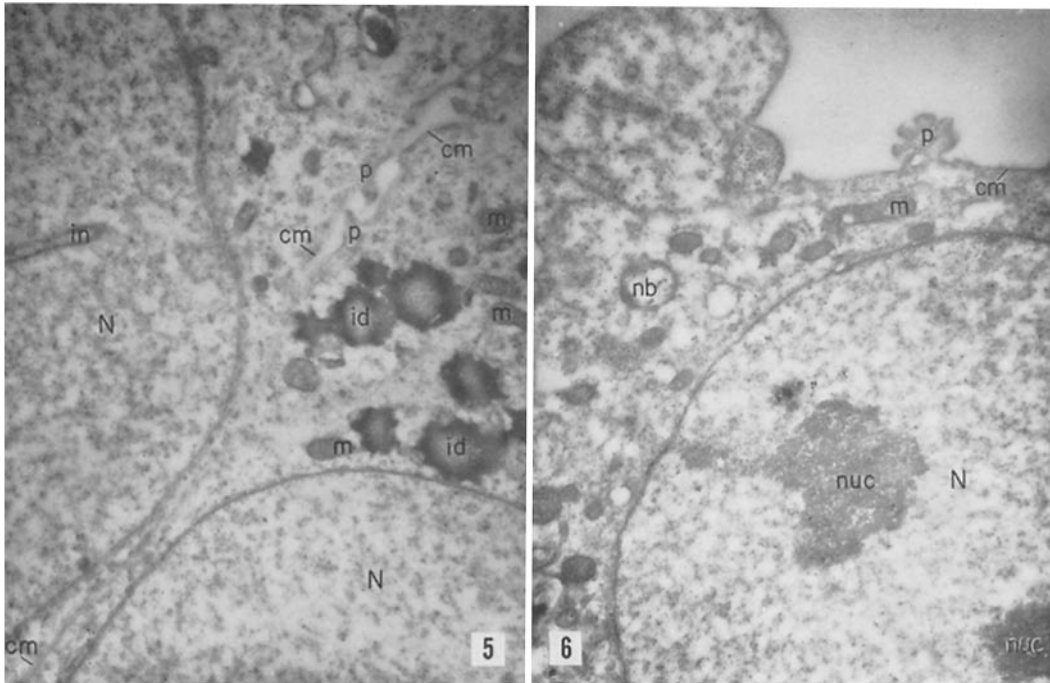
800 A thick) would give rise to tracks. Some random ionization may result through scattering of electrons from the gelatin layer used as a temporary covering for the silver halide particles on the tissue, but this effect would be minimal. In general, only those particles directly overlying a radioactive site are ionized, and these are developed as discrete silver granules, permitting accurate localization of the isotope within the cell.

Thymidine is generally regarded as a specific precursor of deoxyribonucleic acid (10–12), which is localized within the nucleus of the cell. It is therefore remarkable that LLC-MK$_2$ cells incubated with thymidine-H^3 were found to be exclusively labeled in the cytoplasm. No unequivocal explanation can be offered for this, although several published reports have a bearing on the matter.

For example, Plaut and Sagan (13) and Plaut (14) have found that several unrelated strains of *Amoeba proteus* show cytoplasmic incorporation of tritiated thymidine. This incorporation occurs in the presence as well as in the absence of the nucleus, and is independent of the nutritional state of the cells. The effects of incubation of the fixed ameba cells with ribonuclease and deoxyribonuclease show that most, if not all, of the acid-insoluble radioactivity is present in a DNA-like molecule. The authors therefore conclude that, under the conditions of their experiments, there is an accumulation and polymerization of deoxyribonucleotides in the cytoplasm which would normally take place in the nucleus.

However, it is not universally agreed that thymidine is incorporated into deoxyribonucleic acid (DNA). Marshak (15) states that deoxyribonucleotides can be excluded as precursors for the synthesis of DNA, and suggests that either free ribonucleotides or those of the nuclear ribonucleic acid (RNA) are more likely to be utilized. Harper (16) has reported that labeled thymidine remained unaltered when injected subcutaneously into rats, whereas cytidine and uridine were incorporated into nucleic acids. Krause and Plaut (17) maintain that low concentrations of thymidine-H^3 cause serious anomalies in the synthesis of DNA, and Crathorn and Shooter (18) state that autoradiographic activity observed in the nucleus after incubation, washing, and fixing is not necessarily due to thymidine incorporated into DNA. In their view, the labeling is partly due to radioactive precursors which are strongly adsorbed and not easily removed by the fixing and washing procedures.

The labeling shown in Figs. 7 to 15 may therefore be due to adsorption of thymidine-H^3, or a derivative, on some cytoplasmic component, possibly RNA. It is unlikely that thymidine-H^3 would be incorporated as such into cytoplasmic RNA, although certain plant cell ribonucleic acids contain thymine equivalent to about 4 per cent of their uracil content (15).

A further possibility exists that the thymidine might be enzymatically converted to uridine, a known RNA precursor. The labeled uridine would be incorporated into nuclear RNA, which might subsequently appear in the cytoplasm (19, 20),

FIGURE 5

Portions of two adjacent LLC-MK$_2$ rhesus monkey kidney cells prior to coating with photographically sensitive silver bromide particles. One of the nuclei (*N*) has an invagination (*in*). Mitochondria (*m*) and inclusion droplets (*id*) are seen in the cytoplasm. The cell membranes (*cm*) show pseudopodia (*p*). × 11,000.

FIGURE 6

Portion of an LLC-MK$_2$ rhesus monkey kidney cell prior to coating with photographically sensitive silver bromide particles. The nucleus (*N*) has two nucleoli (*nuc*), one of which is in contact with the nuclear membrane. Mitochondria (*m*) and a "nebenkern" structure (*nb*) are seen in the cytoplasm. The cell membrane (*cm*) shows pseudopodia (*p*). × 11,000.

FIGURE 7

Autoradiogram of an LLC-MK$_2$ rhesus monkey kidney cell previously incubated with thymidine-H^3. × 3,500.
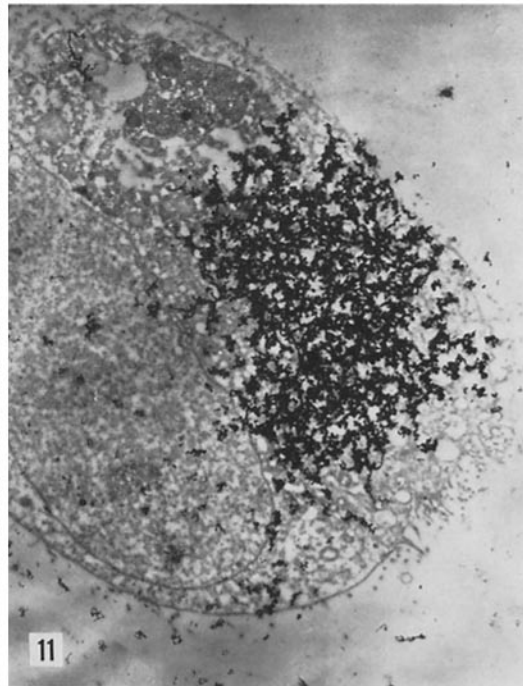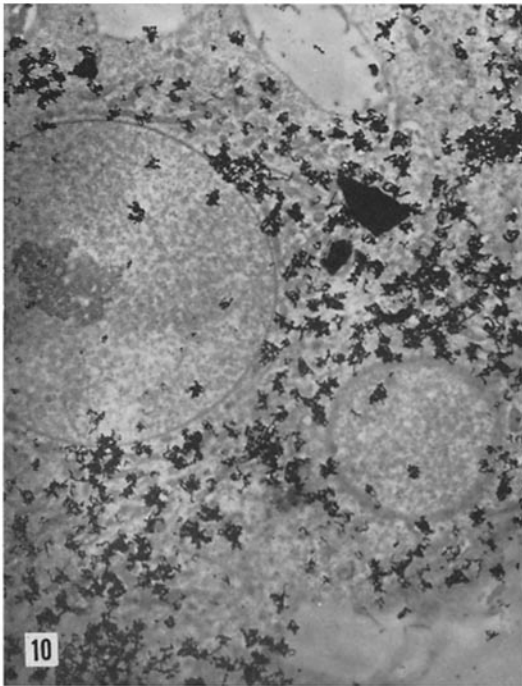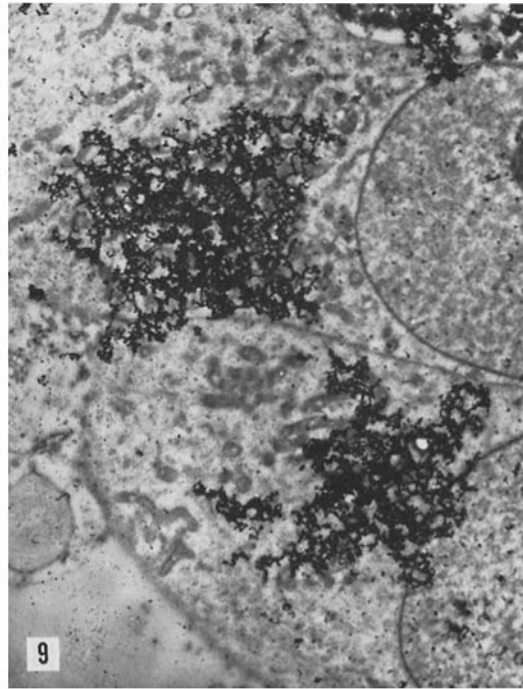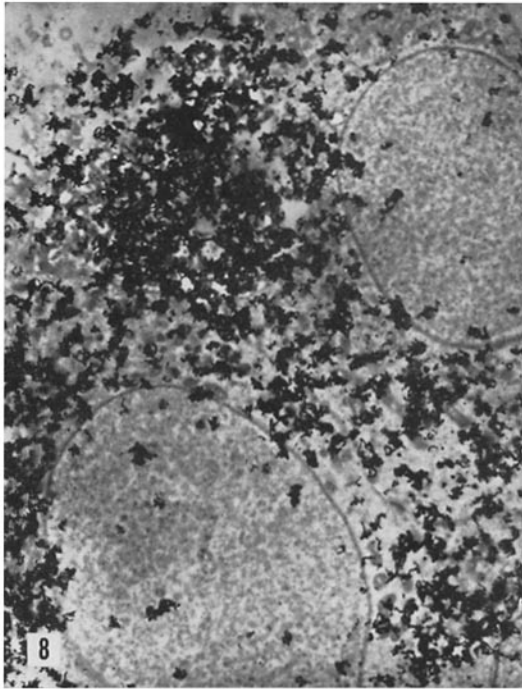
although it is unlikely that the nucleus would be found completely unlabeled under such circumstances.

Any exchange between tritium and labile hydrogen within the cell is also unlikely (11), since autoradiographic activity would then have been detected in the nucleus as well as the cytoplasm.

Although there are indications that small amounts of DNA may occur in mitochondria and microsomes (21) and in the cytoplasm of sea urchin eggs (22), the amounts reported would not be sufficient to account for the degree of labeling shown in Figs. 7 to 15, even if the thymidine content of all such DNA were radioactive.

The possibility that Figs. 7 to 15 may be "autochemograms" produced by the action of reducing substances in the tissue is excluded by the fact that only 60 per cent of the cells were labeled. If chemical action were responsible for the labeling, then all cells would have been covered with a silver deposit. Since 40 per cent of the cells on every grid showed no trace of silver after an identical time of contact with the same photographic emulsion overlying adjacent labeled cells, it is clear that chemical substances in the tissue were not responsible for reduction of the silver bromide. Moreover, it is unlikely that any chemical reducing substances would have been exclusively located in the cytoplasm of the cells which were found to be labeled. Furthermore, osmium fixation and the peroxide catalyst used to polymerize the methacrylate embedding medium would certainly have oxidized any reducing substances in the tissue section.

The work of Smellie, Keir, and Davidson (23) suggests a plausible explanation for the cytoplasmic labeling shown in Figs. 7 to 15. These authors disrupted Ehrlich ascites tumor cells by methods which left the nuclei virtually intact, and then centrifuged the homogenate to obtain a particle-free high speed supernatant fraction. This cytoplasmic fraction was shown to contain a polynu-

cleotide synthetase capable of incorporating thymidine-H^3, together with adenine, guanine, and cytidine nucleotides, into a material which showed the chemical composition and reactions expected of DNA. Bollum and Potter (24–26) and Mantsavinos and Canellakis (27) have also demonstrated the presence of a DNA synthetase in cell free systems prepared from the high speed supernatant fraction of regenerating rat liver homogenates. These results should be accepted with reserve because in dividing cells the nuclear membrane disappears. Thus a DNA synthetase may escape from the nucleus into the cytoplasm and be isolated later in the high speed supernatant fraction of the homogenate. If, however, a polynucleotide synthetase is indeed present in the cytoplasm of nucleated cells, this might account for the cytoplasmic incorporation of thymidine-H^3 shown autoradiographically in Figs. 7 to 15.

Although the site of labeling in Figs. 7 to 15 cannot be readily explained, it is nevertheless apparent that these are true autoradiograms which show a deposit of silver accurately located between the nuclear and cytoplasmic membranes. The technic devised to permit autoradiography in accurate relation to ultrastructural detail at high magnification should prove to have wide application in the field of biochemical cytology.

FIGURES 7 TO 15

Autoradiograms prepared from sections of LLC-MK$_2$ rhesus monkey kidney cells previously incubated with thymidine-H^3. A dense deposit of metallic silver is seen overlying the cytoplasm of each cell. Except for random grains, the nuclei are not labeled. A certain amount of ultrastructural detail has been lost during autoradiographic processing, although mitochondria are clearly visible, especially in Fig. 9. All × 3,500, except Fig. 9, which is × 3,700.
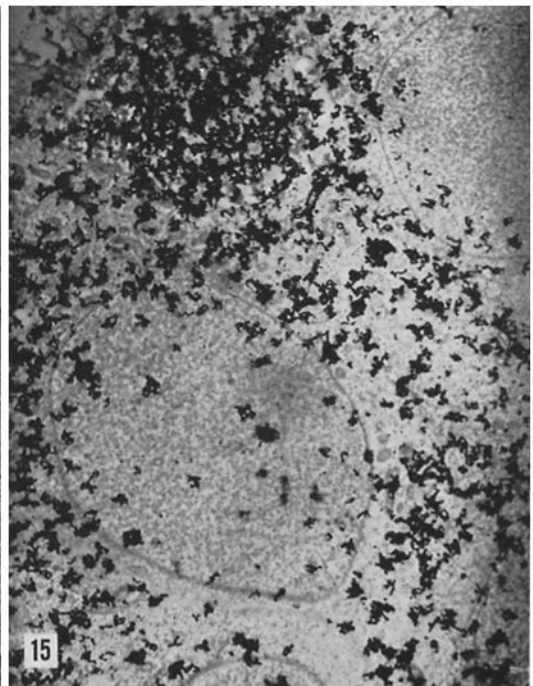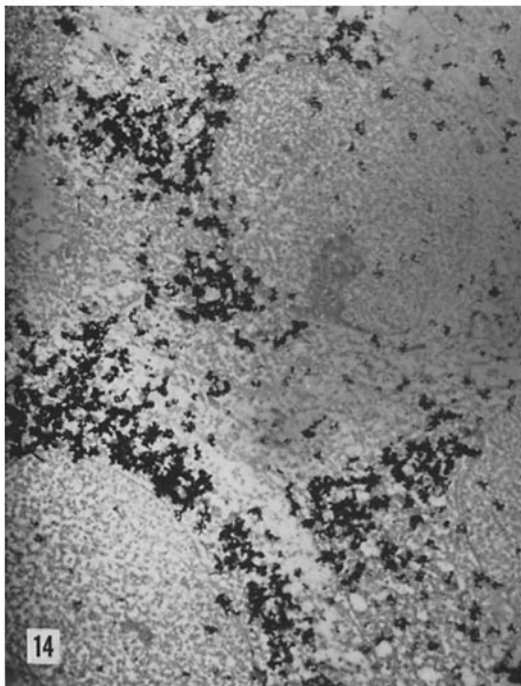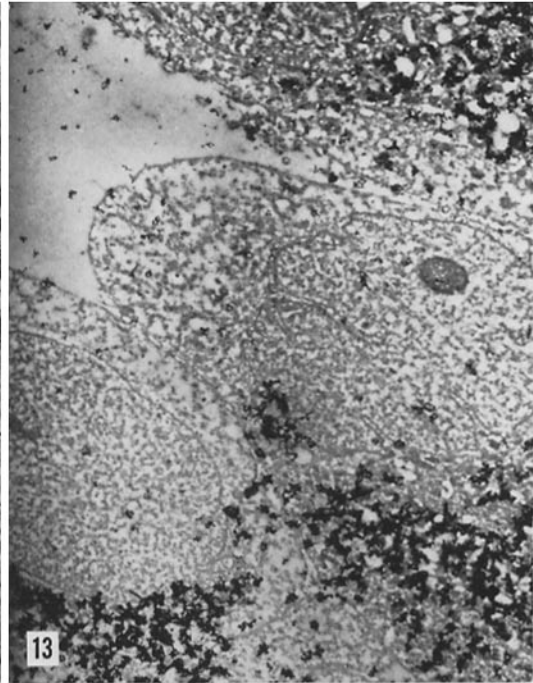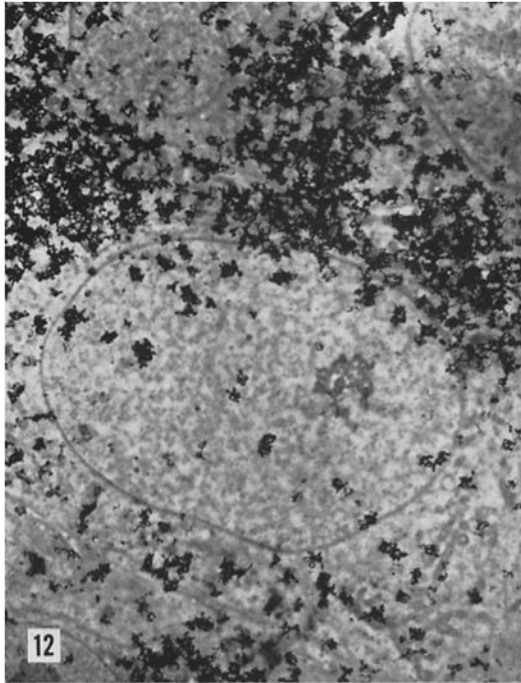
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FIGURES 12 to 15