ELECTRON MICROSCOPIC AUTORADIOGRAPHY

An Improved Technique for Producing Thin Films and Its Application to H^3-Thymidine–Labeled Maize Nuclei

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ABSTRACT

An improved method has been developed for the preparation of autoradiographs for electron optical study. The refinement lies principally in the routine production of uniformly thin layers of photographic emulsion over the tracer-labeled cell sections. This is accomplished by means of a centrifugal spreading mechanism. Maize root tips grown in the presence of H^3-thymidine were examined electron microscopically by this technique and were found to display nuclear labeling with impressive clarity. The new procedure utilized here yields objects in which the finest cell structures are easily resolvable without recourse to gelatin digestion techniques.

INTRODUCTION

The widespread interest in introducing more specificity into fine structure research has fostered several recent attempts to adapt the valuable technique of autoradiography for electron optical use. The pioneering work of Liquier-Milward (4), as well as that of George and Vogt (2) and of O'Brien and George (6), established the feasibility of attempting a union of autoradiography with electron microscopy. These workers also indicated a methodology which has been used with somewhat more refinement in more recent work. These methods basically attempted to use well established, conventional autoradiographic procedures with modifications appropriate to the establish ment of a thin, uniform layer of emulsion over the sectioned or whole cellular material.

Van Tubergen (10), working with H^3-thymidine–fed *E. coli*, prepared the photographic emulsion by gently blowing a loopful of sol emulsion over his bacteria-covered grids. Caro (1) found that the production of an emulsion bubble and its deposit on the specimen grid gave the most satisfactory results. Hampton and Quastler (3) utilized the wire loop method of George to form emulsion but added the refinement of enzymatic digestion of the gelatin after development. Przybylski (8) simply spread his emulsion with a pipette or fine brush and also used enzymatic digestion to obtain convincing micrographs of labeling in H^3-thymidine–fed *Tetrahymena*. Pelc *et al.* (7) used a somewhat complicated procedure of mounting the finished autoradiograph on grids only after all processing was completed, but used a simple dipping method for the establishment of the emulsion film. A novel approach to the production of a photosensitive layer was made by Silk *et al.* (9), who used an evaporated silver film which was later brominated.

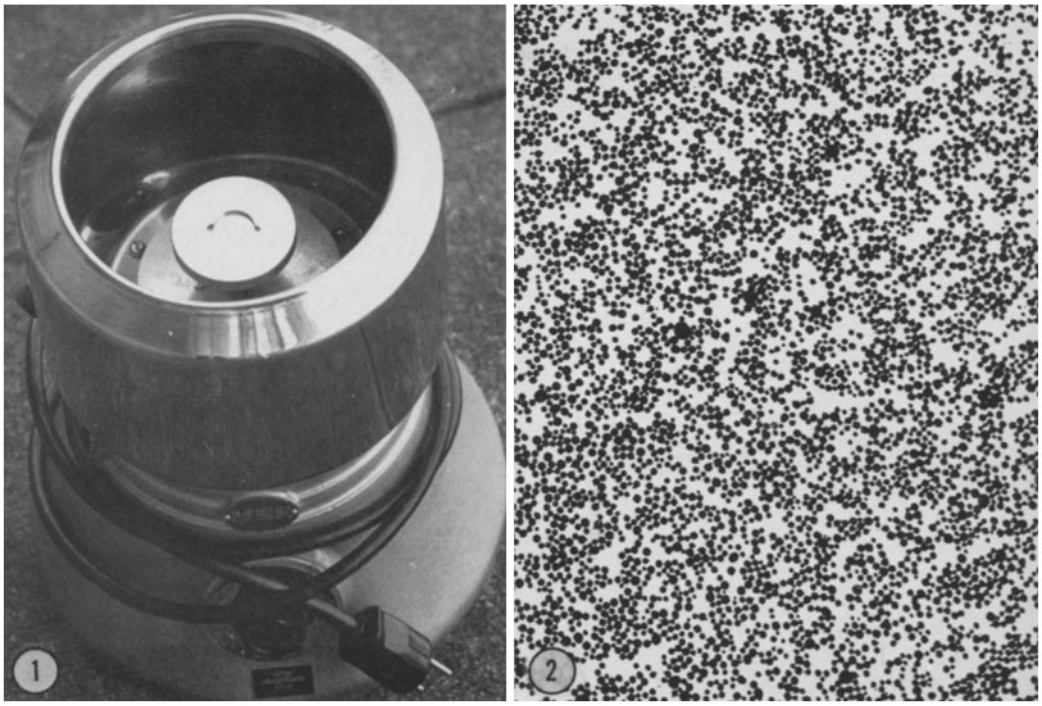


FIGURE 1

Basket-type centrifuge with attached aluminum rotor for coating glass disks with photographic emulsion. Approximately ⅓ true size.

FIGURE 2

Undeveloped G5 emulsion prepared by the centrifugal method, showing uniform and densely packed grains. Centrifuge speed, 10,000 RPM; G5 diluted 1:1. × 4300.

In the work presented here we have attempted by systematic investigation to determine the most satisfactory procedure for establishing the sensitive emulsion over the specimen for electron optical study. Consideration of the problem will lead to the conclusion that this is the crucial first step in any attempt to obtain electron microscopic autoradiographs of good quality.

MATERIALS AND EXPERIMENTAL PROCEDURES

Preliminary Experiments

The initial test objects for our studies were the root tips of 3 to 5 day old maize seedlings which were terminally exposed to a solution containing 10 μc/cc of H^3-thymidine (Schwarz BioResearch, Inc.; specific activity, 0.36 c/mM) for 24 hours. Small slices of root tips were fixed in 1.5 per cent $KMnO_4$ for 1 hour and dehydrated in an acetone series including a 70 per cent acetone bath containing 1 per cent uranyl acetate for contrast enhancement. Araldite-embedded specimens were sectioned with an LKB Ultratome and mounted on carbon-reinforced Formvar-coated grids. Electron microscope observations gave some evidence of precipitated reaction products on copper grids following photographic processing. Nickel and stainless steel grids were therefore employed as well as the standard copper specimen supports. Specimens were examined with a Siemens Elmiskop I electron microscope.

Nuclear emulsions used included Ilford G5 and K2 as well as Kodak V1055. The silver evaporation technique of Silk *et al.* (9) was attempted as a means of obtaining a sensitive film. A new approach utilizing the observation of Marinozzi (5), that $AgNO_3$ impregnates certain tissues and acts as an electron stain, was tested for possible usefulness as an indicator of radioactivity. The procedure was to brominate with vapor or solution the $AgNO_3$-impregnated root tip

sections and to use the AgBr thus produced as a sensitive "emulsion." Unfortunately, very little $AgNO_3$ was found to impregnate our $KMnO_4$-fixed root tip sections. This discrepancy possibly lies in the fact that Marinozzi utilized osmium tetroxide–fixed animal tissues.

Photographic processing was also varied over considerable limits. Home-made "metol" developer as well as Kodak D19 at various dilutions and development times were employed in initial experiments. Fixation was carried out generally in plain nonhardening acid fixer, which was the routine fixation medium of the laboratory.

All the methods utilizing conventional emulsion and application by wire loop, pipettes, bubbles, dipping, etc., were found to yield essentially unreliable results. Films prepared by these techniques varied greatly in thickness and uniformity from one specimen to the next. Occasional good results were achieved by chance, but generally the emulsion was distributed in a blotchy fashion, often taking the form of a honeycomb.

Not all of our problems stemmed from the difficulty of achieving a uniformly thin film over the specimen. Another very disconcerting factor was the bleaching of contrast from our permanganate-fixed material after photographic development and fixation. An essentially negative image could often be observed in the electron microscope due to this bleaching (Fig. 4). This effect could however easily be controlled by the evaporation of a carbon film over the sections mounted on Formvar-coated grids. The carbon evidently provides sufficient protection from the photographic chemicals while not adding enough thickness to the specimen to significantly scatter or absorb the H^3 beta rays.

The difficulties mentioned above tempted us to try the silver evaporation technique of Silk *et al.* (9), which has the inherent advantage of providing a perfectly uniform sensitive layer over the specimen. Although obvious silver grains were observable in such preparations, we were never able to get convincing labeling, but rather always a high "background" of grains. The inherent chemical autosensitivity of such artificially produced "emulsions" might well be expected to yield such "high background" results. This difficulty drove us back to the standard emulsions and a reconsideration of the method of achieving a uniform, thin film.

The Centrifugal Method for Emulsion Distribution

During the course of the experiments detailed above, a technique was suggested to us which in its simplicity of operation and control was an obvious candidate for our electron optical work. This procedure for applying liquid emulsion to specimen grids involves simply the uniform spreading action of a centrifugal field on a fluid drop which strikes the center of the field. A centrifuge head was machined consisting of a rotating aluminum plate containing in its center a small depression just fitting 2 cm glass disks. Fig. 1 shows the MSE basket centrifuge fitted with the rotor. Test experiments indicated that remarkably uniform films resulted from the deposition of a drop of emulsion on the disks while rotating at a high speed. The drops were released from a height of about 5 cm above the rotating disk. The thickness of the emulsion could easily be varied by controlling the dilution (viscosity) of the emulsion and/or the rotational speed of the centrifuge.

Centrifugal theory would obviously not predict that a uniform deposit of emulsion would be obtained with our technique. It is felt that two points in particular make the application of classical centrifugal theory inappropriate with respect to our particular procedure. The first point deals with the extremely rapid drying of films produced by this method. Undoubtedly initial drying occurs immediately as the droplet hits the surface of the spinning glass disk. This effect would tend to counteract a peripheral pile-up of emulsion as predicted from the centrifugal force law. Secondarily, one should mention that the minute size of the specimen (0.01 mm^2), as compared with the total glass disk area of approximately 3 cm^2, would tend to minimize the importance of slight gradients of thickness of the film. In light microscope examinations of many film-covered disks, we could only rarely see significant variation in thickness over the glass disks.

It might be suspected that a fairly high degree of mechanical grain exposure would be caused by this centrifugal procedure; however, background was found to be comparable to or lower than that detected on autoradiographs produced by the more standard techniques.

The basic experimental procedure for emulsion coating, utilizing the centrifugal method, is as follows. Carbon-coated specimens on electron microscope grids are placed face down on a thin Formvar film floating on a water surface. Usually two or three grids are placed centrally and almost in contact with one another. The glass disk fitting the centrifuge rotor is then lowered over the grids with the aid of tongs and picked up in the manner in which grids are routinely coated with Formvar. These steps are shown in the drawings composing Fig. 3. The Formvar film serves the double purpose of anchoring the specimen grids firmly to the glass disks and providing a smooth surface over which the liquid emulsion can spread during centrifugation. All subsequent steps are carried out in a darkroom equipped with a dark red safelight. The thoroughly dry glass disks carrying the specimens are placed on the centrifuge rotor, spun,

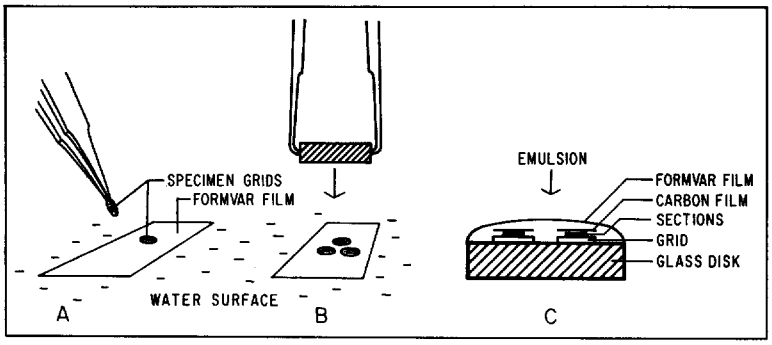


FIGURE 3

Schematic representation of the steps leading to emulsion coating by the centrifugal method. *A:* Grids are placed section side down on a thin Formvar film. *B:* The specimens are picked up from the water surface with a glass disk. *C:* The finished preparation before insertion into the centrifuge rotor for emulsion coating.

coated with a drop or two of liquid emulsion preheated to 45°C, and allowed to spin for approximately 30 seconds after coating to insure drying. The number of drops of emulsion released may be a rather critical parameter, as it was occasionally noted that a second drop did not spread or dry uniformly. It was found that a relatively viscous emulsion (Ilford G5 diluted 1:1) and high rotational speeds gave satisfactory film thicknesses. A speed of 10,000 RPM was routinely used throughout the course of this work. The centrifuge itself was held at room temperature and heating of the rotor due to the brief centrifugation was negligible. The coated glass disks are essentially dry upon removal from the centrifuge and are placed in light-tight cans for exposure. Development after 7 to 14 days was usually carried out in Kodak D19b diluted 1:1 for about 1 minute, followed by a brief rinse with water and fixation for 2 to 3 minutes. Washing for 5 minutes, terminally with distilled water, completed the preparation. Fig. 2 shows an electron micrograph at low magnification of undeveloped G5, made after coating a grid in the manner described. The grains are seen to be densely and uniformly distributed over the area. It should be added that standard copper specimen grids were found to undergo essentially no chemical reactions resulting in precipitate formation when prepared with carbon and Formvar coatings as described above.

OBSERVATIONS

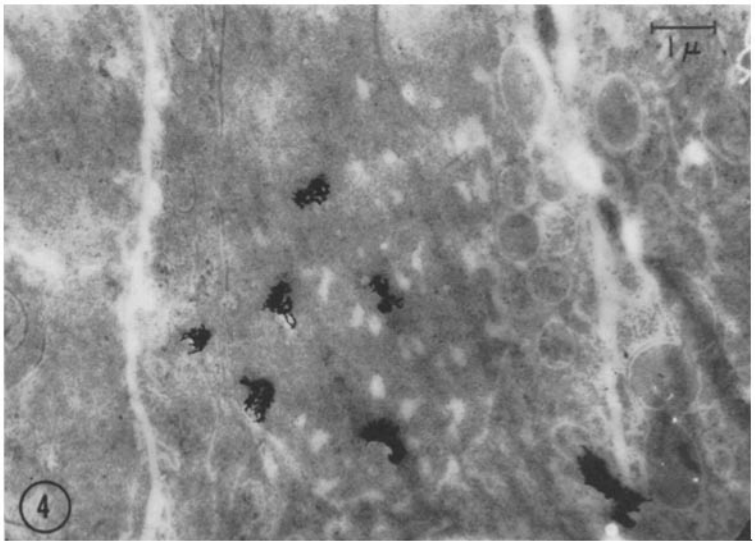
As briefly mentioned earlier, initial preparations of root tips for autoradiography displayed poor preservation of cellular structural detail. Fig. 4 shows such a specimen in which contrast is essentially absent from membrane systems and the cell generally appears to be in poor condition. Even under these adverse conditions, however, nuclear labeling can easily be observed. It was determined that the unfavorable effects on the tissue resulted from the photographic development and fixation during the processing of the autoradiographs. The evaporation of a carbon

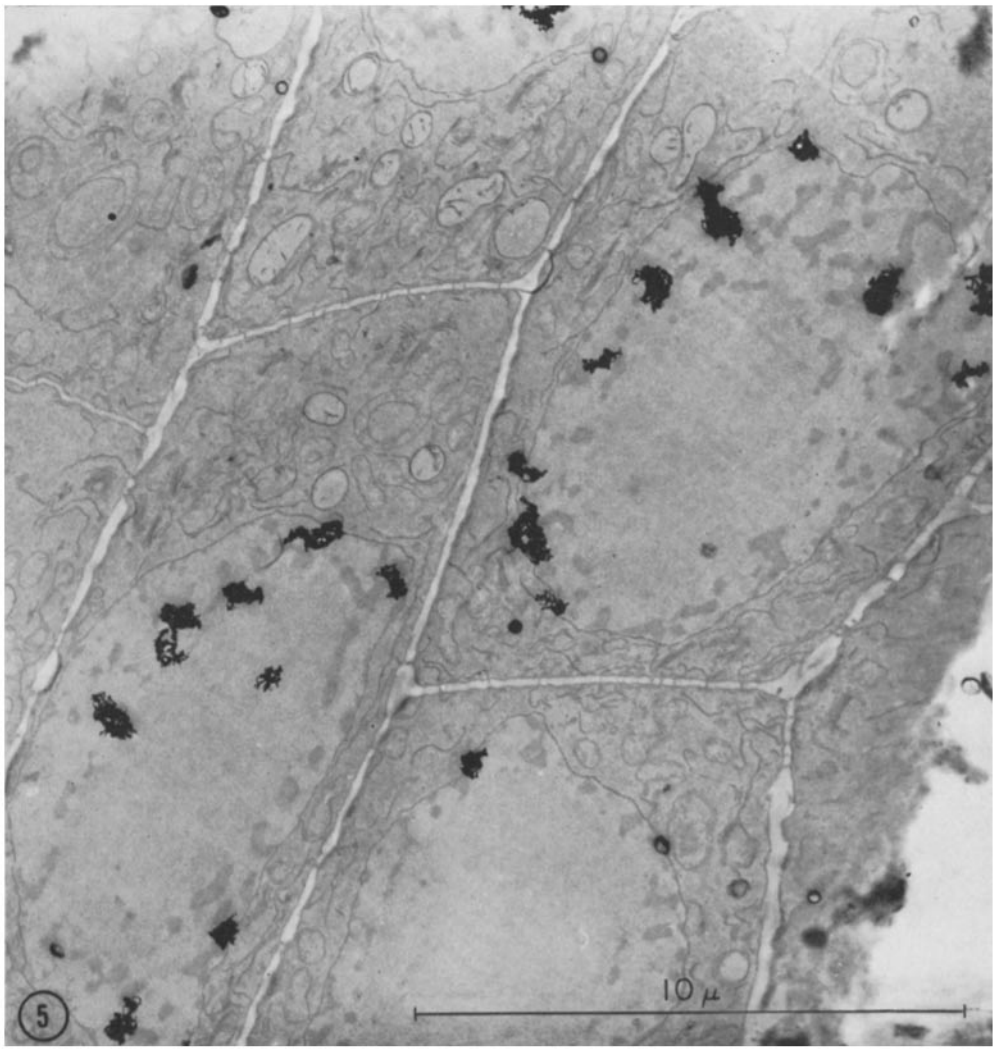
FIGURE 4

Electron microscopic autoradiograph of H^3-thymidine–labeled root tip cell without carbon film protection during photographic processing. Note negative contrast due to the leaching of staining material by photographic chemicals. × 10,000.

FIGURE 5

Survey view of root tip tissue autoradiograph in which the specimen has been protected from photographic chemicals, used in processing, with a carbon film. Silver deposits are localized exclusively in the peripheral nuclear regions of the cells. Ultrastructural detail is very well preserved without gelatin removal. × 7500.





film over the sections was found to be an effective means of protecting them from these undesirable side effects. Fig. 5 shows a survey view of root cells in a section so protected by the improved method of handling. Labeling can easily be observed in two nuclei; essentially no grains appear over the cytoplasm. The rather common occurrence of grains or portions of grains over the nuclear envelope is felt to be the result of geometrical factors between the emanating radiation and the sensitive emulsion rather than of an inherently greater activity in this area. Cell structures are found to be preserved as well as they are in specimens not subjected to the autoradiographic process. The very thin layer of gelatin offers no obvious disadvantage with respect to the resolution of fine structural detail. This can be seen particularly in the higher magnification micrographs composing Figs. 6 to 9, which represent typical portions of root tip cells processed for autoradiography. The rather heavily labeled nucleus in Fig. 6 clearly shows the restriction of activity to the peripheral chromatin as distinct from the central nucleolar area. Figs. 8 and 9 show with particular clarity that grains are predominantly associated with the more electron-opaque areas representing chromosomal material.

It should be mentioned that uranyl acetate–stained controls showed essentially no labeling due to the natural radioactivity of the uranium series. Although undoubtedly a trace number of grains would be expected from this source, the added contrast enhancement from the uranyl stain was thought to outweigh the very slight increase in "background" due to this source.

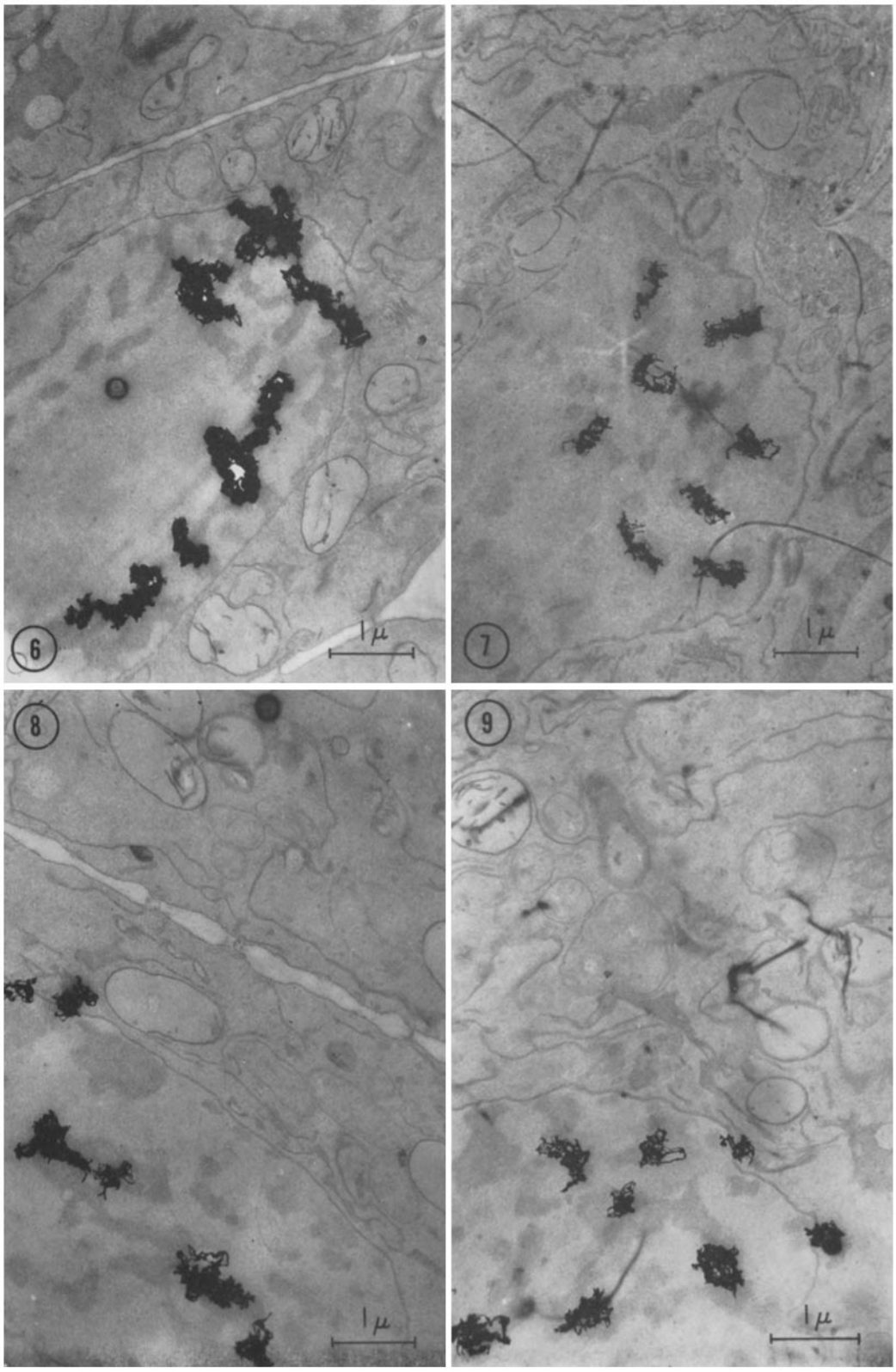
DISCUSSION AND CONCLUSIONS

The speed and simplicity of the centrifugal procedure here described lend it great advantage over the somewhat tedious older methods of applying emulsion for electron microscopic autoradiography. The principal benefit however is the essentially routine manner in which ultrathin, uniform films can be produced. We are no longer faced with the need for enzymatic removal of gelatin which undoubtedly also removes or displaces the photographic grains themselves. The factor limiting the sensitivity of the autoradiographic method is now the large grain size of the exposed silver rather than the adverse properties of poorly applied emulsion. This essentially physicochemical problem is the legitimate stumbling block to be dealt with in utilizing electron microscopic autoradiography, rather than the important technicality of producing a thin and uniform emulsion surface. Our control over the grain size factor is unfortunately limited to the photographic development used when dealing with commercially produced emulsions. Obviously, as short a development time as possible would be expected to produce the smallest grains. This variation was indeed found to exist, but the size spectrum obtainable by simply changing development time is quite small. Specimens developed only 10 or 20 seconds yielded average grain sizes only two or three times smaller than those of objects developed for a few minutes. Some advantage could be gained by utilizing the finest grain developers available. This is no doubt an area where physicochemical research can play an important role, but in the meantime we must attempt to derive what biological information we can from the means at our disposal.

The use of non-hardening fixatives in this work probably resulted in somewhat softer gelatin emulsions during the final wash in photographic processing. It is possible that traces of gelatin were dissolved in this step, leading to a clearer final autoradiograph; however, it should be stated that grain shifting on a significant scale was not observed. It is possible however that certain artifacts such as the small fold-like objects easily visible in Figs. 6 and 8 may be the result of expansion and shrinkage of the gelatin during processing. Detailed experiments comparing the merits of non-hardening and hardening fixation are obviously called for.

FIGURES 6 TO 9

Detail view of several labeled maize root tip cells showing the uniformly good quality of ultrastructure preservation and resolution obtainable with the autoradiographic method described. The silver grains are generally found to be associated with an electron-opaque nuclear component assumed to be chromosomal material. × 13,000.



Even more homogeneous emulsion surfaces can be produced by using more refined grid holders than the glass disks described. The use of metal holders with slight recesses for the grids is one possibility among other types that have been considered.

The results of applying our method to root cells are in a sense trivial since the conclusion that H^3-thymidine is localized in the nuclear chromatin is well known from light microscope and other studies. The importance of this study lies rather in the knowledge that here is an almost routine procedure giving reliable information that will undoubtedly make it possible to obtain answers to more far reaching questions.

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