HIGH-RESOLUTION AUTORADIOGRAPHY

I. Methods

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ABSTRACT

Methods used in obtaining high resolution in autoradiography, with special emphasis on the technique of electron microscopic autoradiography, are described, together with control experiments designed to establish the optimum conditions or procedures. On the basis of these experiments the emulsion selected was Ilford L-4, with a crystal size slightly larger than 0.1 micron. It is applied to the specimen in the form of a gelled film consisting of a monolayer of silver halide crystals. Background, when present, can be eradicated by a simple method. The preparations can be stored, in presence of a drying agent, at room temperature or in a refrigerator. Photographic development is done in Microdol, or in a special fine grain "physical" developer. For examination in the electron microscope the sections are stained with uranyl or lead stains. These methods give a good localization of the label, at the subcellular level, and good reproducibility in relative grain counts.

INTRODUCTION

Autoradiography, used mostly in the past at the tissue or cellular levels, can be brought to the subcellular level and give resolutions of the order of 0.1 μ.

It appears almost obvious that the resolution of autoradiographs will depend upon the thicknesses of the specimen and of the photographic emulsion. Simple theoretical considerations, such as the calculations of Doniach and Pelc (1), confirm this view. By covering thin sections of methacrylate-embedded tissue with thin layers of photographic emulsion, a significant improvement in quality over the classical techniques can be achieved. When the specimen becomes too thin and the photographic grains too small to be seen with light optics, this approach leads naturally to the direct examination of the preparation in the electron microscope.

We shall describe in this paper techniques which lead to high resolution in autoradiography both in the light and electron microscope. During our work with these methods it was often found necessary to perform simple control experiments in order to choose between several possible techniques. The results of a few such experiments will be reported here. A subsequent paper will examine in more detail the problem of resolution in electron microscopic autoradiography. Our aim throughout this work will be resolution rather than absolute quantitation. It should therefore be understood that the techniques used may not always be the best for quantitative work.

METHODS AND RESULTS

1) Choice of Emulsion

GRAIN SIZE: The photographic emulsions commonly used in autoradiography are nuclear

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research emulsions composed, in approximately equal volumes, of crystals of silver halide and of gelatin. Two considerations guide the choice: (a) the emulsion must be capable of registering electron tracks, since most isotopes used in autoradiography are beta emitters, and (b) the size of the silver halide crystals must be small. Among the commonly used emulsions the Kodak (Rochester, New York) NTB and NTB-3 and the Ilford G-5 have a fairly large grain size (0.23 to 0.33 μ) which precludes their use in high-resolution work. The Kodak (London, England) stripping film emulsion AR-10, the experimental emulsion V-1055 (the equivalent of AR-10 in bulk form), and the Ilford Nuclear Research emulsion in gel form K-5 have a grain size of the order of 0.2 μ. The finest grained, electron-sensitive emulsions which we have used were the Ilford L-4, and an emulsion made in the laboratory by Dr. Pierre Demers at the Université de Montréal (2). Both have grains slightly larger than 0.1 μ.

SENSITIVITY: There exists little information regarding the response of various emulsions to β particles from tritium. The sensitivity of nuclear emulsions is usually measured by the number of developable grains per unit distance in the track of particles at minimum ionization. Any such measurement depends, of course, not only on the sensitivity of individual grains but also on their density along the track. The smaller the grain size, the higher the concentration of grains, and therefore the higher the possible number of grains on a track. Studies concerned with autoradiographic responses have usually been made with isotopes such as P^{32}, I^{131}, C^{14} or S^{35} which produce long-range ionizing particles. We have tried to obtain a direct and practical indication of the response to H^3 decays by determining the autoradiographic sensitivity of a number of emulsions. The method of preparation was identical to that described in the next paragraphs. The sources of tritium were cells of *Escherichia coli* fully labeled with leucine-H^3, that is to say, grown in presence of leucine-H^3 for more than seven generations. The relative sensitivity was determined by counting the average number of grains per cell (29). A total number of at least 300 cells on three different slides was counted for each emulsion, the average grain count per cell varying between 2 and 4 grains. Exposure was at room temperature, over Drierite. Development was in D-19 for 2 minutes at 20°C, except

for L-4 for which it was 4 minutes. A number of such experiments were performed and in each case the response of K-5 was used as a base line and arbitrarily set at 100. Table I summarizes the results.

On the basis of these results L-4 was chosen as the most suitable emulsion for electron microscopic autoradiography. For light microscopic autoradiography, it has the disadvantage of

TABLE I

Grain Size and Relative Sensitivity of Various Nuclear Emulsions

The grain diameter was measured in the electron microscope and is accurate to $\pm$ 10 per cent. The relative sensitivity was measured by taking the average grain count per cell in autoradiographs of bacteria labeled with tritiated leucine. Development was for 2 minutes, in D-19 at 20°C, except for L-4 (4 minutes). The differences in autoradiographic response reflect, in part, differences in grain size, since an emulsion with large silver halide crystals has fewer of them per unit volume.

Emulsion	Grain diameter	Relative sensitivity
	μ	
Ilford L-4	0.12	132
Demers	0.12	61*
Ilford K-5	0.18	100
Kodak V-1055	0.17	51
Kodak AR-10	—	57
Kodak NTB-3	0.23	48
Kodak NTB	0.27	—
Ilford G-5	0.32	—

* This value was obtained without sensitization. When the emulsion was sensitized with triethanolamine, as specified by Demers (2), the sensitivity became equivalent to that of L-4.

showing frequent tracking (several grains caused by one decay) when used in thick layers with tritium. This complicates grain counts in quantitative work. (This tracking largely disappears, for reasons which will appear later, when the emulsion is applied as a monolayer of crystals, such as is obtained in electron microscopic preparations.) For this reason we have usually preferred K-5 for light microscopic preparations on the basis of its high sensitivity, low tracking, and similarity in composition with L-4.

2) Specimen Preparation

The preparation of the labeled specimen is identical to that of ordinary material for electron microscopy: fixation in buffered osmium tetroxide, dehydration in graded alcohols, embedding in methacrylate or epoxy resin, sectioning on an ultramicrotome (24). The blocks are cut somewhat larger than usual to give a better sampling of the specimen. It is always advisable to verify that the incorporated label is preserved during the preparation. This is done by sampling each processing solution and counting the amount of released tritium in a scintillation counter. In some previous experiments (3, 4) we had found, for example, that with bacteria labeled either fully or with a pulse of tritiated leucine, uridine, cytidine, or thymidine, the loss was extremely low. But any new situation should be investigated in this respect.

For autoradiography at the phase-contrast microscope level, methacrylate is the preferred embedding material. Methacrylate sections as thin as 0.2 to 0.3 micron can be seen clearly in phase contrast when the embedding medium is removed. This cannot be done conveniently with epoxy resins and much thicker sections are needed to provide sufficient contrast. Clean microscope slides are prepared (subbed) by dipping in a solution of 0.1 per cent gelatin and 0.01 per cent chromium potassium sulfate, and drying in a vertical position. Sections are cut at a thickness of 0.4 micron, picked up with a pointed wooden applicator, and floated on a drop of water placed on the center of the slide. They can then be expanded with xylene vapors (5) and the slide is dried at 40°C. The methacrylate is removed by a 10-second dipping in amyl acetate.

For electron microscopic autoradiography, thin sections (pale gold) are picked up on a screen coated with a collodion film backed by a thin carbon layer. The section must be perfectly flat and smooth. This also holds for the collodion film and the grid supporting it. Electroplated grids, such as the Athene models, have been found particularly good in this respect. After drying, the screens are attached by a small portion of their edge to a small piece of double-coated masking tape (Scotch No. 400) fixed to a microscope slide. Three to four grids, close to each other, can be put on each slide. In all these operations cleanliness is essential.

3) Preparation and Application of the Emulsion

The emulsions K-5 and L-4 come in gel form and have the shape and consistency of short pieces of cooked spaghetti. They can be handled under yellow-green light. A 10-minute exposure of a slide coated with K-5 to a 25 watt safelight with filter AO at a distance of 2 meters does not significantly increase the background. It is best, however, to keep the exposure to light at a minimum. Before use, the emulsions are normally stored in the refrigerator. They have a limited shelf life and after a while background builds up to very high levels. The useful life of an emulsion is quite variable but in general we have used K-5 up to 2 months and L-4 up to 4 months after the date of manufacture indicated on the bottle. To avoid possible complications (postmaturation, high background, etc.) emulsions which have been melted and diluted are used only once and discarded.

For light microscope preparations, we have used a simplified version of the dipping method of Messier and Leblond (6). To prepare the emulsion, 20 grams of K-5 are put in 20 ml of distilled water in a Coplin jar and melted at 45°C for 15 minutes. After stirring gently, but thoroughly, with a glass or plastic rod, the emulsion is cooled at room temperature (23°C) for 30 minutes. The slides are dipped and withdrawn with a slow and uniform motion. The excess emulsion is drained and the slides set to dry in a vertical position. A gentle stream of warm, filtered air helps to accelerate the process. When the emulsion begins to dry, it appears quite uniform and without streaks, provided the slide was not dirty (finger grease, etc.).

For electron microscopic preparations the applied emulsion should consist of a monolayer of silver halide crystals, closely and uniformly packed over the specimen (Figs. 1 and 3). Microscopic examination, after drying, of emulsions applied in the sol state reveals that the grains are distributed in a non-random manner over the specimen and outline the surface irregularities (Fig. 2). Another common defect of this method of application is that the thickness of the emulsion is very uneven due to drying stresses. To avoid these defects the emulsion must be applied after it has gelled, as suggested by van Tubergen (7).

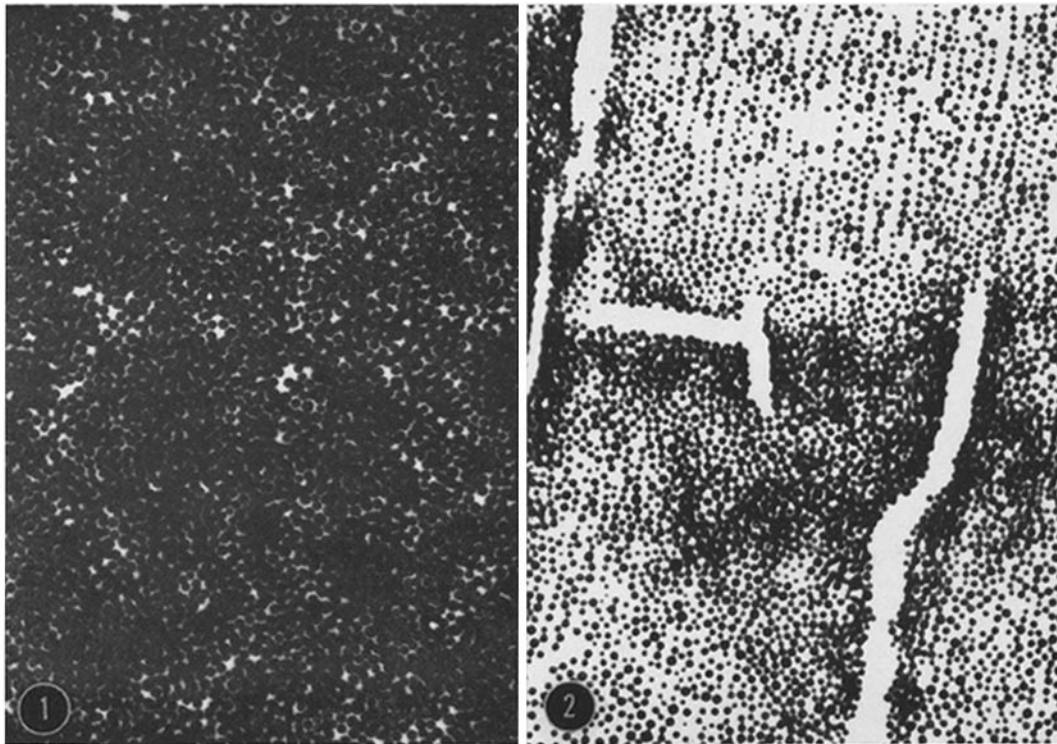FIRST METHOD: Ten gm of Ilford L-4 are

FIGURE 1

L-4 emulsion applied over a thin tissue section using the second method described in the text. Because of the high contrast of the image the section is not visible. The uniformity of the distribution of the silver halide crystals is good and the defects in the pattern are random with respect to the specimen. This uniformity is preserved over the entire area of the specimen screen. × 8000.

FIGURE 2

L-4 emulsion applied over a thin tissue section using the loop method, but while the emulsion is still in the liquid state. The pattern formed by the crystals is very irregular and it follows the surface defects of the section. (Sections of poor quality were used in both preparations.) × 7000.

melted in 20 ml of distilled water in a 300 ml beaker at 45°C for 15 minutes. After thorough stirring, the beaker is placed in an ice-bath for 2 to 3 minutes, then at room temperature for 30 minutes. The emulsion has now become very viscous. A loop of thin wire (platinum, silver, or copper), 4 cm in diameter, is dipped in the emulsion and withdrawn slowly, forming a thin film in the loop. If the preparation is correct this film gels almost immediately. The loop is then touched to the surface of the slide and the film falls on the grids and adheres to them very firmly. If the gelling of the film does not take place rapidly, or if microscopic examination reveals gross unevenness

in the distribution of silver halide crystals, the time of cooling in the ice-bath is increased slightly.

SECOND METHOD: Even when the film is applied in the gel state some redistribution of grains is likely to occur. Although the procedure outlined above gives generally good results for routine work, a more elaborate method was used when very even distributions were needed for quantitative work. A 2 per cent solution of purified agar (Difco) in distilled water is poured to a thickness of 0.5 cm in Petri dishes. After hardening it is stored in a refrigerator. Before use the agar plate is removed from the dish and rectangles 2 × 3 cm are cut and placed on microscope slides

with the agar surface, which was in contact with the dish, facing up. These slides are warmed to 37°C for a few minutes to remove surface moisture. The agar blocks are then flooded with 0.2 per cent Parlodion in amyl acetate and dried in a vertical position. Subsequent manipulations are done in the dark room. An emulsion is prepared as in the previous method, using 10 gm of L-4 to 40 ml of water, and simply cooled to room temperature. The loop is used to form a film which

brought under the collodion-emulsion membrane and lifted out of the water. After drying they are attached to a glass slide as described above. This method, when used with sections, has the disadvantage of interposing a thin collodion membrane between the emulsion and the specimen, thus reducing slightly the sensitivity and the resolution. With minor variations it is particularly useful for the preparation of particulate specimens (*e.g.* viruses, etc.).
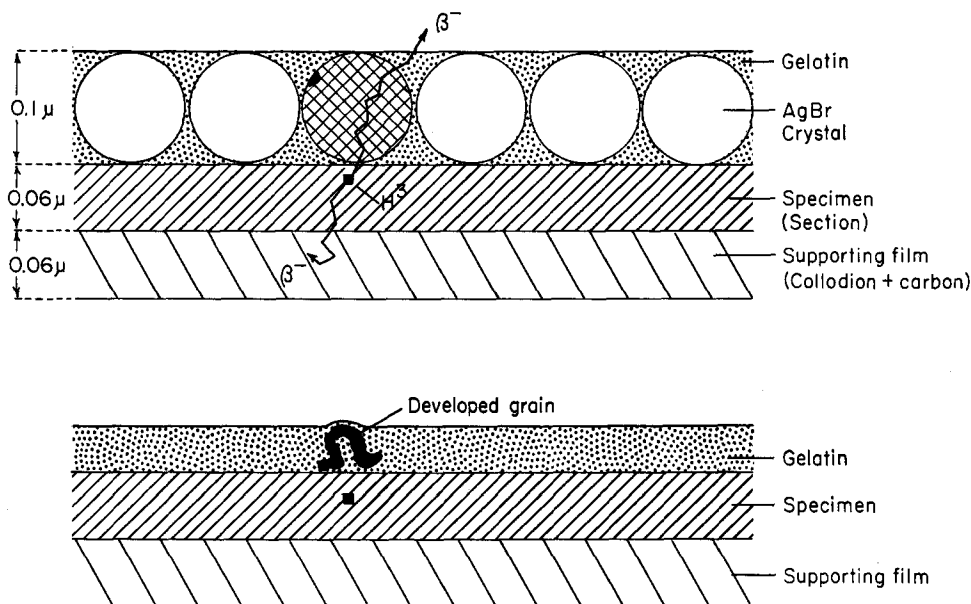

FIGURE 3

Diagrammatic representation of an electron microscope autoradiograph preparation.

Top. During exposure. The silver halide crystals, embedded in a gelatin matrix, cover the section. A beta particle, from a tritium point source in the specimen, has hit a crystal (cross-hatched) causing the appearance of a latent image on the surface (black speck on upper left region of crystal).

Bottom. During examination and after processing. The exposed crystal has been developed into a filament of silver; the non-exposed crystals have been dissolved. The total thickness has decreased because the silver halide occupied approximately half the volume of the emulsion.

is applied to the collodion-agar surface without waiting for gelling. Because of the smoothness of the surface and of the diffusion of the water from the emulsion to the agar (through the collodion membrane), drying artifacts are prevented and very uniform distributions of silver halide crystals are obtained. The collodion-emulsion membrane is then floated on a water surface (emulsion side up). Thin sections of the material to be examined have been previously picked up on grids without a supporting membrane. They are placed on a piece of fine-meshed metal screening in the water,

Although the methods described here were designed for high resolution rather than quantitation, their reproducibility is satisfactory. For example, in one experiment at the light microscope level, grain counts made over seven slides of labeled bacteria, using K-5 emulsion, gave an average grain count per cell of 3.30 ± 0.34, *i.e.* a standard deviation of the order of 10 per cent, which is comparable to results obtained with stripping film emulsions (9). In the electron microscope, relative grain counts over various regions of cells are also quite reproducible. As an example,

Reproducibility of Grain Counts Made over Various Regions of Pancreatic Exocrine Cells,
for 4 Different Preparations from the Same Block

In this specimen, DL-leucine-H³ was injected intravenously and the pancreas was fixed 20 minutes later. For more details on this work see references 22, 23.

Although a certain amount of biological variation is probably superimposed on the methodological error, the reproducibility of such relative grain counts is good and well within the range of expected statistical variation. (For the first line $\chi^2 = 0.479$, $P \cong 0.80$).

	Section number	
Region of the cell	1	2	3	4	Average
Golgi	74%	71%	78%	72%	73%
Zymogen	15%	10.5%	3%	11%	10%
Rough endoplasmic reticulum	7.5%	9%	16%	12%	11%
					Total grains
Total grains counted	67	208	100	144	519

Table II gives the relative grain counts over various cellular structures for four different preparations of pancreatic exocrine tissue, 20 minutes after intravenous injection of tritiated leucine. Another test for the linearity of the autoradiographic response is the distribution of grain counts over uniform specimens. We have shown that cross-sections of bacteria uniformly labeled with uridine-H³ provide such a specimen, and that a Poisson distribution of grain counts over them is obtained with stripping film emulsions (4). The situation remains true at the electron microscope level, as shown in Table III.

4) Background Eradication

Background is almost never produced during exposure if proper precautions are taken. It is sometimes present in the emulsion, especially with K-5, and builds up with time. We have routinely developed test slides for all preparations, immediately after the emulsion was dry. If the background was objectionable it was removed in the following manner, derived from a method proposed by Yagoda (10): The bottom of a 3 × 4 inch staining dish is covered with five thicknesses of filter paper wetted with 10 ml of freshly made 3 per cent hydrogen peroxide; the slides to be eradicated are placed on a glass holder in the dish, taking care that they do not touch the wet paper; the dish is covered tightly and placed in a light-proof box. The slides are exposed to H_2O_2 vapors for 3 to 5 hours, then dried thoroughly to eliminate all traces of H_2O_2. Fig. 4 shows the effect of this

Autoradiographic Grain Counts over Thin Cross-Sections of B. subtilis Labeled with Uridine-H³ in an Electron Microscope Preparation

The fact that a Poisson distribution is obtained shows that the autoradiographic response is fairly uniform over the various areas surveyed (50 fields in three thin sections). This compares favorably with results obtained in light microscope preparations with stripping film emulsion (4).

Number of grains	Number of sections	Expected from Poisson
0	219	223
1	184	176
2	72	69.5
3	18	18.3
4	1	4
\multicolumn{2}{c	}{$\chi^2 = 0.936$}	$P > 0.5$

treatment on background and sensitivity. A treatment of 5 to 6 hours decreases the background by 95 per cent and leaves the sensitivity unaffected. A 24-hour treatment results in a slight reversal of this effect. This treatment will oxidize and eradicate the latent image of autoradiographic grains as well as of background grains and therefore can only be applied at the beginning of the exposure. It should be established that the incorporated label is not affected by H_2O_2. Thus we found that, in osmium-fixed bacteria or in

pancreatic tissue, exposure to H_2O_2 did not affect incorporated leucine-H^3, uridine-H^3, and thymidine-H^3. This was shown by the fact that treatment in H_2O_2 did not affect the grain count over
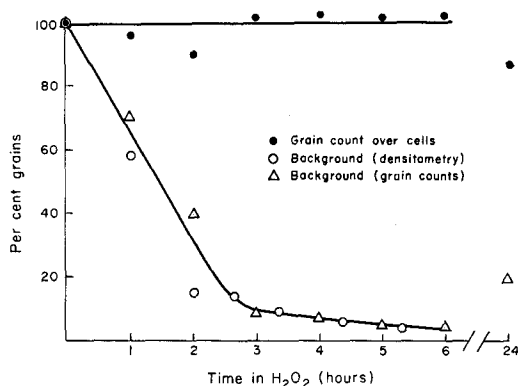
FIGURE 4

Background eradication in H_2O_2 atmosphere. Preparations of *E. coli* labeled with leucine-H^3. Background was measured as number of grains per 100 μ^2 or by densitometry. In both cases the values were measured relatively to the value at time 0. The sensitivity, measured as the average grain count per cell, does not change during the first 6 hours of treatment, while the background decreases by 95 per cent. All slides were given the same exposure after eradication.

the cells, nor did it cause release of radioactive material into the emulsion. The possibility of creating an artifact should not, however, be ignored. It is known, for example, that osmium-fixed lipids are solubilized by H_2O_2 (11) and it was found that incorporated choline-H^3 was released from osmium-fixed membranes and diffused into the emulsion (Dr. David Luck, private communication), causing a decrease in grain counts and an increase in apparent background.

5) Storage and Exposure

The storage conditions during exposure will affect: (a) the sensitivity of individual crystals, (b) the regression of the latent image, and (c) the background. The second of these effects has received the most attention and it is generally accepted that regression (that is to say the disappearance of the latent image from a previously exposed crystal) is due to an oxidation of the silver speck constituting the latent image and can be minimized by storage in CO_2 or nitrogen (12, 13) and storage at low temperature (14). We have measured the over-all autoradiographic response of K-5, and L-4, under various storage conditions (by counting grains over fully labeled bacteria). The results, shown in Table IV, seem to differ from what might be expected from the regression

TABLE IV

Effect of Various Storage Conditions on the Over-All Autoradiographic Response of K-5 and L-4 Emulsions

The grain count per bacterial cell has been normalized with respect to the value found for storage in air at 4°C over Drierite (set arbitrarily at 100). The cells used in the various experiments had different amounts of label and the average grain counts varied from 1.5 to 3 grains per cell. In each series all slides were developed together in D-19 at 20°C for 2 minutes, except for the L-4 emulsion which was developed 4 minutes. Each point represents the average of 300 cells on 3 different slides.

The fact that, in the K-5 experiments, the differences between various storage conditions are less pronounced with longer exposure times might indicate that the length of the exposure is also an important factor in determining the best storage conditions.

Storage conditions					
Temperature	−20°C	4°C	4°C	4°C	20°C
Atmosphere	Air	CO_2	Air	Air	Air
Drying agent	Drierite	Drierite	Drierite	$ZnCl_2$*	Drierite
Emulsion and exposure time					
K-5 (2 days' exposure)	36	60	100	112	136
K-5 (5 days' exposure)	83	80	100	100	116
K-5 (7 days' exposure)	100	106	100	—	121
L-4 (7 days' exposure)	85	114	100	—	140

* Excess $ZnCl_2$ in contact with saturated solution, giving approximately 10 per cent relative humidity.

effect alone (12, 14). Storage in CO_2 does not seem to affect markedly the sensitivity, while temperature has a definite effect. Other emulsions may, of course, show different results.

It is possible that in our preparations, with a thin well dried emulsion exposed to slow and therefore highly ionizing electrons, the regression of the latent image is not an important factor and the major effect of storage conditions is on the sensitivity of individual crystals. On rare occasions background has been found to increase for the storage conditions which resulted in higher overall sensitivity, but in general it is not affected by any of the conditions used here for periods of as long as 3 to 4 months. In practice the dried slides are stored with a small pack of Drierite in Bakelite slide boxes and in a light-proof cardboard box, and kept at room temperature. Storage in a refrigerator, over Drierite, might be preferred if there is a possibility of chemical reactions between specimen and emulsion, or in extreme climatic circumstances.

The exposure time depends on the amount of label incorporated, its distribution in the specimen, and many other factors. It can therefore be predicted only on rare occasions, and is usually determined by developing test slides at regular intervals. Because of the thinness of the layers, the electron microscope method has extremely low sensitivity. We used a simple rule to estimate the necessary exposure in the type of preparations described above: if a light microscope preparation gives a suitable autographic response in 1 week, a useful preparation for the electron microscope will need a 2 to 4 months' exposure. The sensitivities of the two methods differ therefore by a factor of 10, approximately.

6) *Photographic Processing*

DEVELOPMENT OF LIGHT MICROSCOPE PREPARATIONS: A photographic developer reduces rapidly to metallic silver grains the exposed silver bromide crystals which carry a latent image. The non-exposed grains are also affected but the reaction takes place with some delay. If it is interrupted early enough by placing the slides in a solution of sodium thiosulfate (hypo) the non-exposed crystals are dissolved, leaving only the reduced grains in the emulsion. The number of developed image grains (from exposed crystals) increases rapidly at the beginning of the reaction, then reaches a plateau (2), the only subsequent

change being an increase in size (15). The behavior of background grains is opposite: they remain at a fairly constant level at the beginning; then increase in number very rapidly as the reaction progresses (2). For practical purposes we tried to determine the conditions of development which give a maximum number of grains without an undue increase in background. This was done for L-4 and K-5, using tritium-labeled bacteria to measure the autoradiographic response, and developing the preparations in D-19 at 20°C
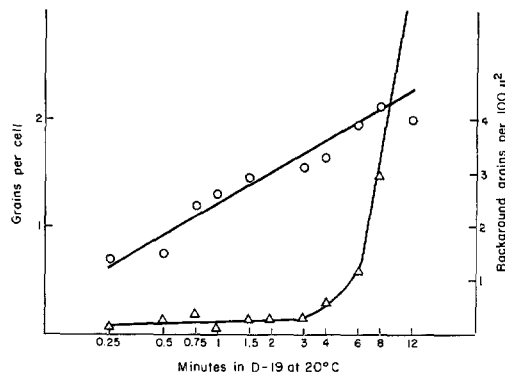

FIGURE 5

Development curve for K-5. The grain counts were made on preparations of *E. coli* labeled with leucine-H³. The average grain count per cell increases with developing time. The background remains low for the first 3 minutes of development, then rises suddenly. A 2-minute development time gives low background and good sensitivity. Since the slope of the grain count per cell curve has a low value (notice that the time scale is logarithmic), reproducible results can be obtained.

(Figs. 5 and 6). Similar curves for the stripping emulsion AR-10 have been shown by van Tubergen (9). Photographic processing of slides for light microscopic autoradiography was done in glass staining dishes, ten slides being processed in one operation. All solutions were kept at 20°C.

In the case of K-5 we found that a plateau in the grain count was not reached before the background becomes objectionable.[1] The time of development was therefore arbitrarily chosen at 2 minutes and a careful control of temperature and time of development was exercised when the

[1] We have found recently that by adding Kodak anti-fog No. 1 (benzotriazole) to the D-19, in the recommended amount, a plateau could be reached at 6 to 8 minutes of development, while keeping a very low background.

reproducibility of the results was important. For L-4 a plateau was reached after approximately 2 minutes, and a 4-minute development time was found to give good results.

The optimal processing conditions derived from such experiments are summarized for various situations in Table V.
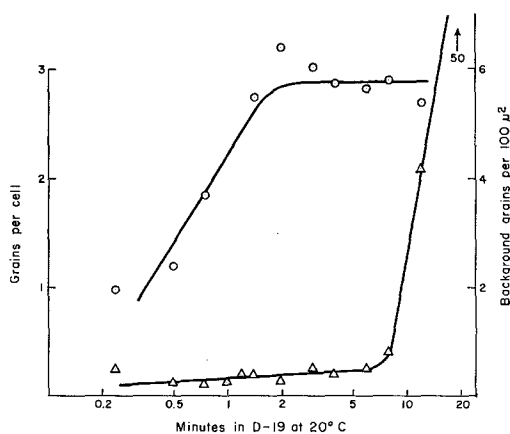

FIGURE 6

Development curve for L-4. The grain count per cell reaches a plateau after 2 minutes in D-19 at 20°C, while the background does not rise significantly until 6 to 8 minutes. The development time chosen was 4 minutes.

DEVELOPMENT OF ELECTRON MICROSCOPE PREPARATIONS: We can also apply the information gained from the type of development curves described above directly to the electron microscope level. There, however, we encountered other problems more directly related to high resolution, the first one being the relation between the original event, the passage of a charged particle through a crystal of silver halide, and the final event, the grain of silver which we observe in the microscope.

Following the passage of a charged particle through a crystal of silver halide a latent image, probably formed by a few molecules of reduced silver (2), appears on the surface of the crystal. There does not seem to be a direct relationship between the position of this latent image and the path of the particle. For example, we have found that a single hit from a beta particle from tritium could produce as many as three distinct developable latent images in one single crystal (see later). There is therefore a certain amount of uncertainty in the localization of a β decay inherent to this phase of the process and which does not seem capable of being improved except by reduction of the size of the crystals.

The next step is the growth, in the developer, of a silver grain, by reduction of silver ions, in a

TABLE V

Schedule of Photographic Processing for Various Types of Preparations

All solutions are kept at 20°C. Slides which have been stored in the cold should be warmed to room temperature before processing. Edwall Quick Fix has also been used as a fixer.

| Type of preparation	Developer	Stop	Fixer	Rinse
Light microscope K-5	D-19, 2 min.	1% acetic acid 10 sec.	Kodak rapid fixer 5 min.	Running water, 5 min. Distilled water, 1 min.
Light microscope L-4	D-19, 4 min.	1% acetic acid 10 sec.	Kodak rapid fixer 5 min.	Running water, 5 min. Distilled water, 1 min.
Electron microscope L-4	Microdol, 5 min.	1% acetic acid 10 sec.	Kodak rapid fixer 5 min.	Running water, 5 min. Distilled water, 1 min.
Electron microscrope L-4 (fine grain)	Physical developer* 1 min.	1% acetic acid 10 sec.	Kodak rapid fixer 5 min.	Running water, 5 min. Distilled water, 1 min.

* See text.
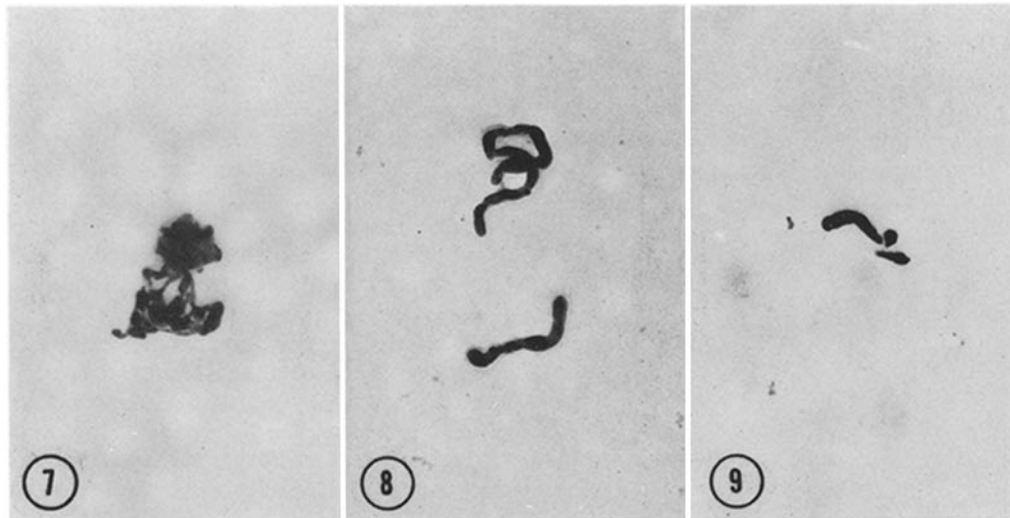
FIGURE 7

Grain of L-4 emulsion, exposed by a tritium decay, and developed in D-19 at 20°C for 2 minutes. Tritiated leucine was mixed with an emulsion which was applied as a monolayer of crystals. Exposure was calculated to give a low grain count. The developed grain appears as a large, complex and filamentous structure. × 45,000.

FIGURE 8

Grains of L-4 emulsion, prepared as in Fig. 7, and developed in Microdol-X for 5 minutes. The exposure was higher than in Fig. 7. The grains have a simple structure, usually consisting of a single filament. The position of the grain is defined as the median point on a straight line joining its extremities, since it is impossible to know which one was the starting point. × 45,000.

FIGURE 9

Grains of L-4 emulsion, exposed as in Fig. 7, and developed in the fine grain developer, described in the text, for 1 minute at 20°C. Three distinct latent images have been developed from a single hit on one crystal. Notice the possible variations in the sizes of the developed grains. The common point of all three grains, indicating the position of the original crystal, seems to be at their pointed end. × 45,000.

reaction which is believed to be catalyzed by the silver speck of the latent image (15). There are two basic types of developer, known traditionally, and without any clear reason, as "chemical" developer and "physical" developer.

CHEMICAL DEVELOPERS: A chemical developer, such as D-19, reduces the silver halide crystal itself. The final result is a long filament of silver which grows out of the surface of the crystal and coils randomly in a process that is not well understood (15) (Fig. 7). This coil can have, in the case of L-4 developed in D-19, a diameter of 0.3 to 0.4 micron. A fine grain developer such as Microdol-X (Eastman Kodak) produces in general a single strand of silver, rather than a coil

(Fig. 8). There does not seem to be any good way to decide which end of this filament originated on the silver halide crystal. We therefore used the middle point of a line drawn between the two extremities of the filament as the estimated position of the original latent image. This imposed, on the average, an error on the position of a single grain which, in our estimation, is less than 1000 A. In cases when the highest possible resolution is not required this has been found to be quite acceptable. We have therefore used Microdol-X as a routine developer for electron microscopic autoradiography.

Other fine grain developers have been tried, in particular Ansco 110, Ansco Finex-L, Unibath, a

chlorohydroquinone developer proposed by Demers (2), and a hydroquinone developer proposed by Loveland (quoted in 15). In all cases the grains were longer and more complex than with Microdol-X. In some cases they were thinner but this did not result in higher resolution. When a dilute developer is used, as proposed by Przybylski (16), or when the temperature of the developer is lowered, the over-all sensitivity decreases and the filaments are not shortened but they become much thinner. This has a major disadvantage: either spontaneously or under the electron beam such long, thin filaments tend to disintegrate into a number of small silver particles giving only the appearance of a finer grain without really improving resolution since they originate from the familiar filamentous form.

PHYSICAL DEVELOPERS: To arrive at a better definition of the position of the latent image we relied on the method of "physical" development described by Lumière, Lumière, and Seyewetz (17). We used the prefixation type (15) in which the developer dissolves the silver bromide crystals, leaving only the latent image upon which silver ions present in the solution are then attached. The best formula among the variations which we have tried was a solution of 0.1 M sodium sulfite and 0.01 M paraphenylenediamine as the reducing agent (the sodium sulfite is dissolved first in water at 50°C after which paraphenylenediamine is added. The final solution is filtered before use). This solution is not stable and should be freshly made. The variations tried, and discarded, were changes in the ratio of the two components and addition of various amounts of silver nitrate. With a development of 1 minute, at 20°C, the resulting grains are extremely small, either spherical or comma-shaped (Fig. 9). It can be demonstrated that the pointed end of the comma corresponds to the origin of the grain (see below). Therefore, either because the grain is small or because we know its orientation with respect to the original speck of silver, we can define the position of the latent image that produced it with an error which we estimate at approximately 200 A.

Although this developer gives good resolution and sensitivity, it is unstable and less reproducible than Microdol-X. In particular the size of the grains, while generally small, is difficult to control. We use it, therefore, only when the highest possible resolution is required.

RELATION BETWEEN BETA DECAYS AND PHOTOGRAPHIC GRAINS: A few control experiments were performed in order to obtain a better understanding of the relation between beta decay and photographic grain. In one such experiment leucine-H³ was mixed with the emulsion, which was then applied as a uniform monolayer of crystals. The exposure was calculated to give less than one grain per 25 μ^2, so that the probability of having two distinct registered decays separated by less than 0.5 μ was close to zero. After development in Microdol-X the preparation was scanned for the presence of tracks (two or more grains separated by less than 0.5 μ). It was found that only 1.8 per cent of the grains were double, and no tracks of more than two grains were found. Since approximately 30 per cent of the decays from tritium would have enough energy to expose more than one grain, it seems clear that, in a monolayer of silver bromide crystals, a hit by a beta particle on a crystal reduces the probability of another hit, by the same particle, on a second crystal to an almost negligible value. This is probably an important contribution to resolution (see following article). The situation would be quite different if there were several layers of grains. Indeed with the thicker emulsions used at the light microscope level, tracks from tritium decays as long as 4 to 6 grains are commonly found in L-4.

When the physical developer already described was used to develop a similar preparation (emulsion to which leucine-H³ has been added) it was found: (a) that the number of developed grains was comparable to that obtained with Microdol; (b) that approximately 6 per cent of the grains carried two latent images and gave rise to two developed grains very close to each other, while approximately 1 per cent had three latent images and produced three grains; (c) that when several grains grew from separate latent images on the same crystal their origin (assumed to be the point at which the separate grains are closest to each other) was always the point of the comma.

We have no direct information concerning the sensitivity of individual crystals. It seems likely, though, that the probability that a crystal hit by a tritium beta will produce a developed grain is fairly close to one. The response of K-5 to beta decays from tritium was found to be approximately one grain per decay (unpublished observations). Since tracks are very rare, this implies that one hit

on a crystal gives a grain. In the case of L-4, tracks are more frequent but the number of grains per decay is higher than one so that the situation with respect to individual crystals is approximately the same.

FIXATION AND WASH: The other steps of the photographic process cause little difficulty. Fixation in Kodak acid fixer occasionally fixed the gelatin in a coarse pattern which obscured the phase-contrast image. Rapid fixers such as Kodak rapid fixer or Edwall Quick Fix gave generally better results. Fixation and wash should be complete and the use of fresh and clean solutions is mandatory.

7) Final Steps

After photographic processing the light microscope slides are dried thoroughly and stored in plastic slide boxes. For examination a large coverslip is mounted with a drop of water or a solution of glycerin (the contrast of the image can be modified by varying the concentration of glycerin) and the slide examined in phase contrast with an oil immersion objective. After examination the coverslip is removed, the slide washed in distilled water, dried, and stored. With reasonable care this process can be repeated many times without damage to the slide. Slides have been stored in this manner for 2 years without deterioration. Mounting of the coverslip with one of the usual permanent mounting media results in an almost complete loss of contrast because of the high index of refraction of these media.

The gelatin of the emulsion does not usually interfere very much with the phase contrast image. If needed, it can be cleared to a considerable extent, without effect on the grains, by treatment of a few minutes in 0.05 N NaOH (see below).

Electron microscope preparations can be stained after the photographic processing. Since the

presence of a gelatin layer over the specimen reduces the contrast of the image a strong staining is indicated. We have found that 1 per cent uranyl acetate mixed before use with absolute alcohol to give a final concentration of 30 per cent ethanol (18) and applied for 10 to 45 minutes gave good results. Lead stains can also be used but it should be remembered that many formulae are strongly alkaline and will tend to remove the gelatin (19).

Removal of the gelatin has been practiced by several authors as a means to enhance contrast of the final image. The procedures used include proteolytic digestion (20, 16), alkaline digestion (19), warm water after fixation in a non-hardening hypo (21). In our experience all these procedures have the disadvantage of causing, on occasion, a displacement of the grains and almost always some loss of grains. The most useful procedure seems to be that of Revel (19), which combines lead staining and clearing of the gelatin and which we have found to cause only rarely a displacement of the grains. To insure against possible artifacts we have done all grain countings and measurements on specimens in which the gelatin layer had been preserved, and have removed the gelatin only to improve the quality of images used for illustration. None of the preparations used to illustrate this article or the following one was treated to remove gelatin. When the gelatin remains in place the specimen is relatively thick and we have used high electron microscope voltages (80 or 100 kv) to minimize damage and reduce chromatic aberration which is particularly noticeable because of the thickness of the preparation.

Some examples of the results obtained are shown in Figs. 10 to 12 in which *Bacillus subtilis*, labeled with uridine-H³ or with thymidine-H³, was used as a test specimen. The resolution obtained is clearly sufficient to resolve the nuclear region of these small cells ($\sim 0.6 \times 2$ micron).
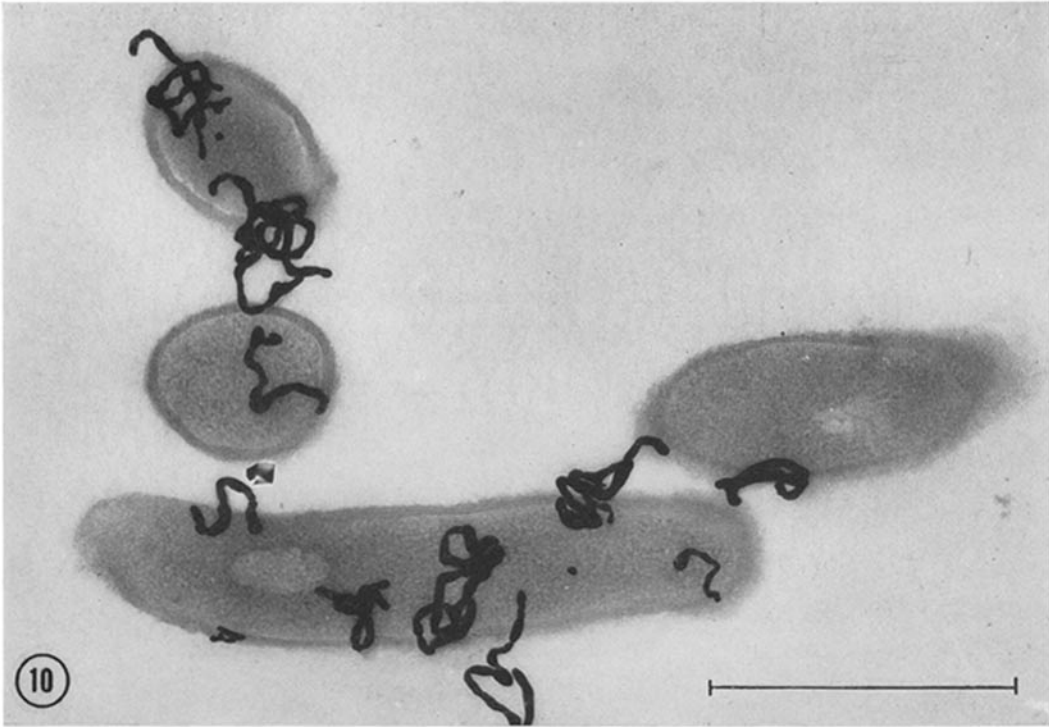

FIGURE 10

Autoradiograph of thin sections of *B. subtilis* labeled fully with uridine-H³. Stained in uranyl acetate. Development in Microdol-X. There is no obvious localization of the label. $\times$ 40,000.

FIGURE 11

Thin sections of *B. subtilis* labeled fully with thymidine-H³. Stained in uranyl acetate. Development in Microdol-X. The grains seem closely associated with the nuclear region. $\times$ 38,000.
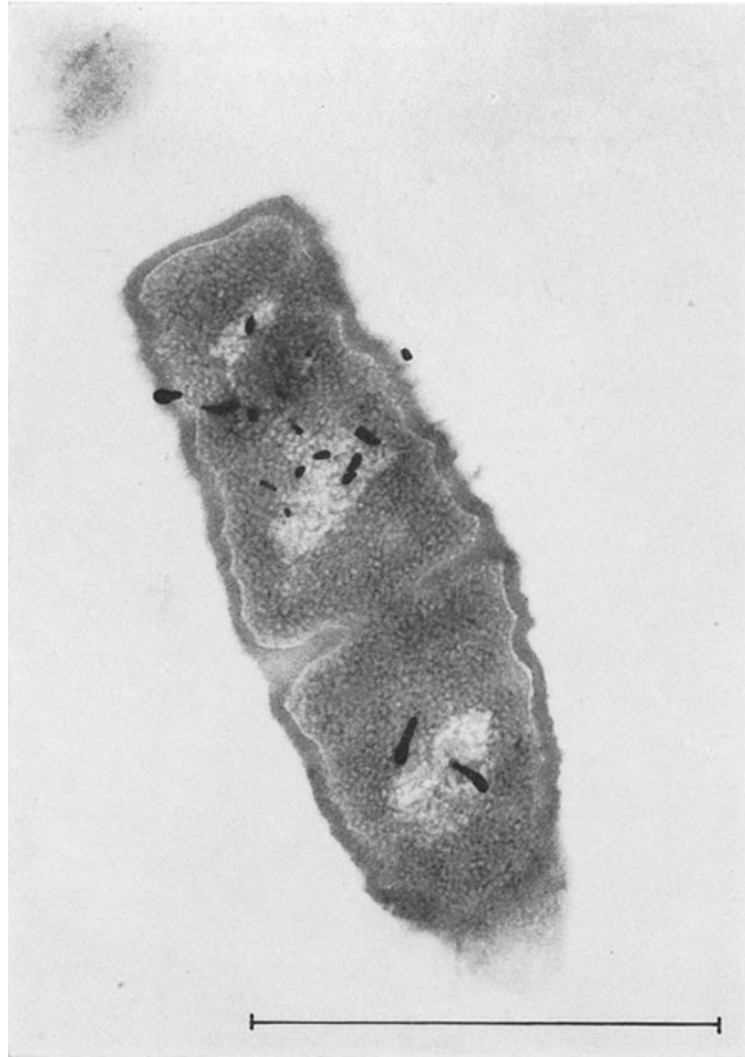
FIGURE 12

Thin sections of *B. subtilis* labeled fully with thymidine-H³. Stained in uranyl acetate. Development in fine grain "physical" developer. Notice small size and good localization of the grains with respect to the nuclear regions. × 58,000.

DISCUSSION

The procedures used in autoradiography are simple and the interpretation of the results usually straightforward if one has a reasonable understanding of the various steps leading to the final image. Such an understanding is especially important for the achievement of satisfactory results in the newly developed technique of electron microscopic autoradiography. We have described in this article methods which we and other workers in this laboratory have applied successfully to a number of problems. Whenever it seemed appropriate we have also described the control experiments which led to the selection of a particular step. These descriptions are intended only as a guide toward a workable technique and it should be understood that almost every step is probably susceptible of considerable improvement.

The most critical operation is the application of the photographic emulsion. Most of the methods

described previously (16, 19, 20, 22, 25–27) involved the application of a liquid film of emulsion. As we have seen, this often results in an irregular distribution of the silver halide crystals. The two methods described here consist in the application to the specimen of a preformed, gelled, film of emulsion, thereby avoiding a displacement of the grains with respect to the surface features of the specimen. They give consistent results, the second method giving more uniformity, at the cost of a more complicated handling and a small loss in resolution.

The method of Silk *et al.* (21), consisting in the bromination of an evaporated film of silver, seems to offer some advantage with respect to uniformity. However, the results obtained by these authors suggest strongly the possibility of an artifact, since they find grains grouped in large clumps and exclusively over the cytoplasm of tissue culture cells labeled with thymidine-H^3. It is possible that, because of the intimate contact between the specimen and the unprotected silver bromide crystals, chemical reactions can take place readily between them and result in reduced grains. In spite of this, it is possible that a modification of this technique might produce good results.

Absolute quantitation, in autoradiography, is always difficult, as pointed out by Levi (28). This is especially true in electron microscopic autoradiography since it is very difficult to know exactly the distribution of silver halide crystals over the specimen. Relative results can, however, be obtained without too much difficulty and in a reproducible manner. It should be obvious that grain counts should be made only on photographs of randomly selected fields.

On the basis of our experience with the method of electron microscopic autoradiography and of the experiments described in this article we have reached the following general conclusions:

(*a*) Because of its small grain size and high sensitivity to electrons Ilford L-4 Nuclear Research emulsion seems to be the most suitable commercial emulsion.

(*b*) Specimen preparation does not present any special problem but the thin sections should be prepared with more than routine care.

(*c*) The emulsion should be applied in such a way that a monolayer of silver halide crystals is formed and that the distribution of these crystals is uniform and not affected by the specimen. Two methods giving an approximation of a monolayer are described.

(*d*) Background is usually low at the electron microscope level but when present it can be eliminated by a simple method described in the text.

(*e*) Preparations should be stored with a drying agent either at room or refrigerator temperature.

(*f*) Photographic processing is a critical step. For routine applications Microdol-X was found to be a good developer. For the highest possible resolution a special developer, of the physical type, is preferred. Its formula and mode of application are given in the text.

(*g*) The sections should be stained in order to obtain sufficient contrast. This is done, through the gelatin, after photographic processing. Uranyl acetate is employed most frequently. Alkaline lead stains have been used occasionally to improve image quality (19), but they should be used cautiously since they partially remove the gelatin and might disturb the position of grains.

A detailed discussion of the problem of resolution and of the advantages of the electron microscopic method of autoradiography will be found in the following article (*8*).

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