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Assessment of resolution by half distance values for tritium and radioiodine in electron microscopic radioautographs using Ilford L4 emulsion developed by "Solution Physical" or D-19b methods

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Summary. Half distance values for electron microscopic (EM) radioautographs with the isotopes ^{3}H and ^{125}I were determined using Ilford L4 emulsion processed with either fine grain, solution physical development, or filamentous grain, chemical development with D-19b. ^{3}H- and ^{125}I-line sources, obtained by cutting perpendicular sections from sections of ^{3}H-labeled methacrylate or ^{125}I-labeled thyroid glands, were processed for EM radioautography. The distribution of silver grains around a line source was determined by measuring their distance from the source in photographs of EM radioautographs. The number of silver grains per unit distance from the line source was plotted on graphs and half distance values were calculated. With solution physical development, the half distance value was 76 nm for ^{3}H and 80 nm for ^{125}I; whereas with D-19b development it was 187 nm for ^{3}H and 157 nm for ^{125}I. Since solution physical development produced a reduction of about 50% in the half distance values for both isotopes, it is concluded that the production of fine grain by this method provides better resolution for EM radioautography than filamentous grain development with D-19b.

Introduction

In electron microscopic (EM) radioautography, resolving power is frequently estimated by measuring the half-distance (HD) value, that is, the distance from a thin radioactive line source within which 50% of the silver grains are located (Salpeter et al. 1969). Most of the methods devised to statistically determine the relative content of radiolabel among biological structures from the distribution of silver grains in the radioautographs, depend on the knowledge of the HD, or some variant thereof (Salpeter et al. 1969, 1977, 1978; Williams 1969; Nadler 1971, 1979; Blackett and Parry 1973, 1977). In practice, HD values are often taken from published data (Blackett and Parry 1977; Salpeter et al. 1978). However, HD values may easily vary according to experimental conditions, such as section thickness, type of isotope, type of emulsion, method of application of emulsion and photographic development. Accordingly, unless identical conditions are used, HD values assessed in another laboratory may not always be appropriate.

Hence, HD values were determined for the most frequently used conditions for electron microscopic radioautography with Ilford L4 emulsion in this laboratory. A line source for the isotopes ^{3}H and ^{125}I was prepared and the HD values were measured after chemical development with D-19b that produces filamentous silver grains or solution physical (SP) development that produces fine grains.

Materials and methods

1) Preparation of a thin radioactive line source

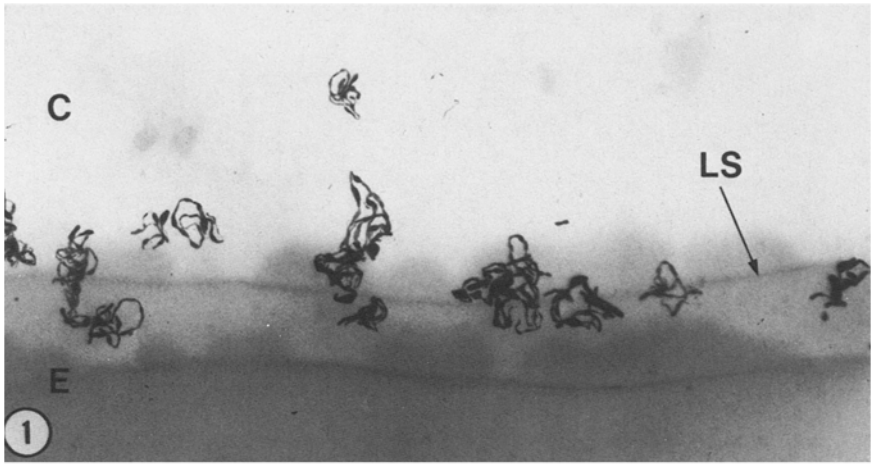
The radioactive source for tritium was a block of poly-n-butyl methacrylate homogeneously labeled with 10 mCi ^{3}H per g (Amersham Corporation) and for ^{125}I, Epon embedded rat thyroid glands labeled at a concentration of 10 μCi ^{125}I per g body weight. Rats had been sacrificed 24 h after intraperitoneal injection of ^{125}I by perfusion with 2.5% glutaraldehyde and thyroid glands were then postfixed by immersion for 2 h in 2.5% glutaraldehyde followed by 2 h in 1% OsO_4, dehydrated in alcohol and propylene oxide, and embedded in Epon.

Preparation of profile sections. At first, sections of ^{3}H-labeled methacrylate and ^{125}I-labeled thyroid were cut at recorded thickness of 50 nm on a LKB ultramicrotome and placed on the slot of formvar coated slotted grids. The grids were then reembedded in flat moulds on the surface of Epon with the section in a horizontal position facing upwards (Yang and Shea 1975). After Epon had polymerized, the copper grid was trimmed away and the section was recut at a 90° angle to its surface. The resulting profile sections of the original radioactive section provided a 50 nm line source of radioactivity.

2) Electron microscopic radioautography

Profile sections of pale gold interference color placed on celloidin coated slides were coated with Ilford L4 emulsion by means of a semiautomatic coating instrument which provides identical monolayers of densely packed silver bromide crystals on all specimens (Kopriwa 1973). After 20 days exposure in dry air at 4° C

* This work was the subject of a McGill University dissertation (Levine 1977)



Figs. 1 and 2. Radioautographs showing the distribution of silver grains along a radioactive line source, obtained from profile sections of a 50 nm thick section of ^{3}H-labeled methacrylate coated with Ilford L4 emulsion. *LS*, line source; *E*, Epon side of line source; *C*, celloidin side of line source. ×25,000 (bar = 1 μm)

Fig. 1. After development with D-19b silver grains are large and filamentous

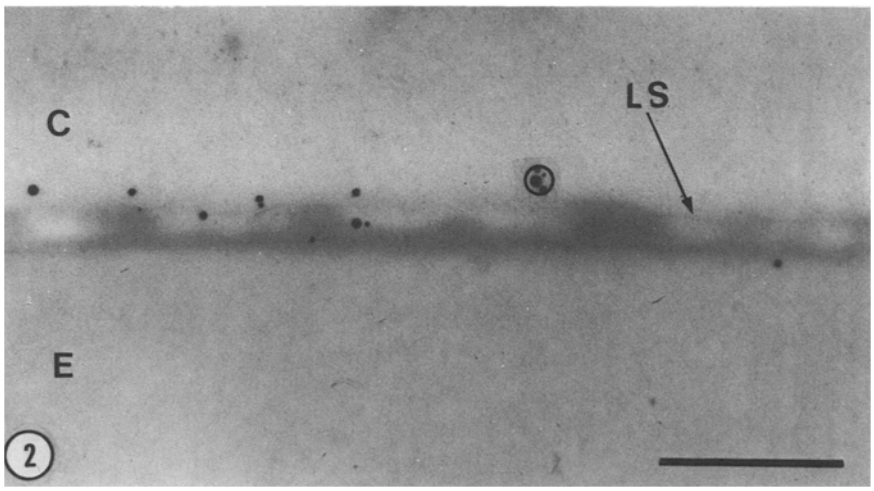


Fig. 2. After development with solution physical developer, silver grains are small spherical silver deposits, which occur either singly or in clusters of two or more. A circle of the mean diameter of the silver bromide crystals is placed over a cluster of silver deposits to determine the center of the grain

for ^{125}I and 63 days for ^{3}H, radioautographs were developed by the two principal development procedures used in this laboratory for routine EM radioautography. To obtain filamentous silver grains, development was for 1 min in D-19b developer diluted 1:10 with distilled water. To obtain small, compact silver deposits, development was for 7 min in Agfa-Gevaert solution physical (SP) developer preceded by 1 min latensification in gold thiocyanate (Kopriwa 1975).

The distribution of silver grains around a line source, obtained with the 4 different isotope-development combinations (^{3}H-D19b, ^{3}H-SP, ^{125}I-D19b, ^{125}I-SP) was examined in a Hitachi HU-11C electron microscope. Micrographs, each demonstrating 4 to 30 silver grains, were taken along the length of the line source at ×10,000 magnification until a total of approximately 500 grains was accumulated for each experimental group, with one exception.

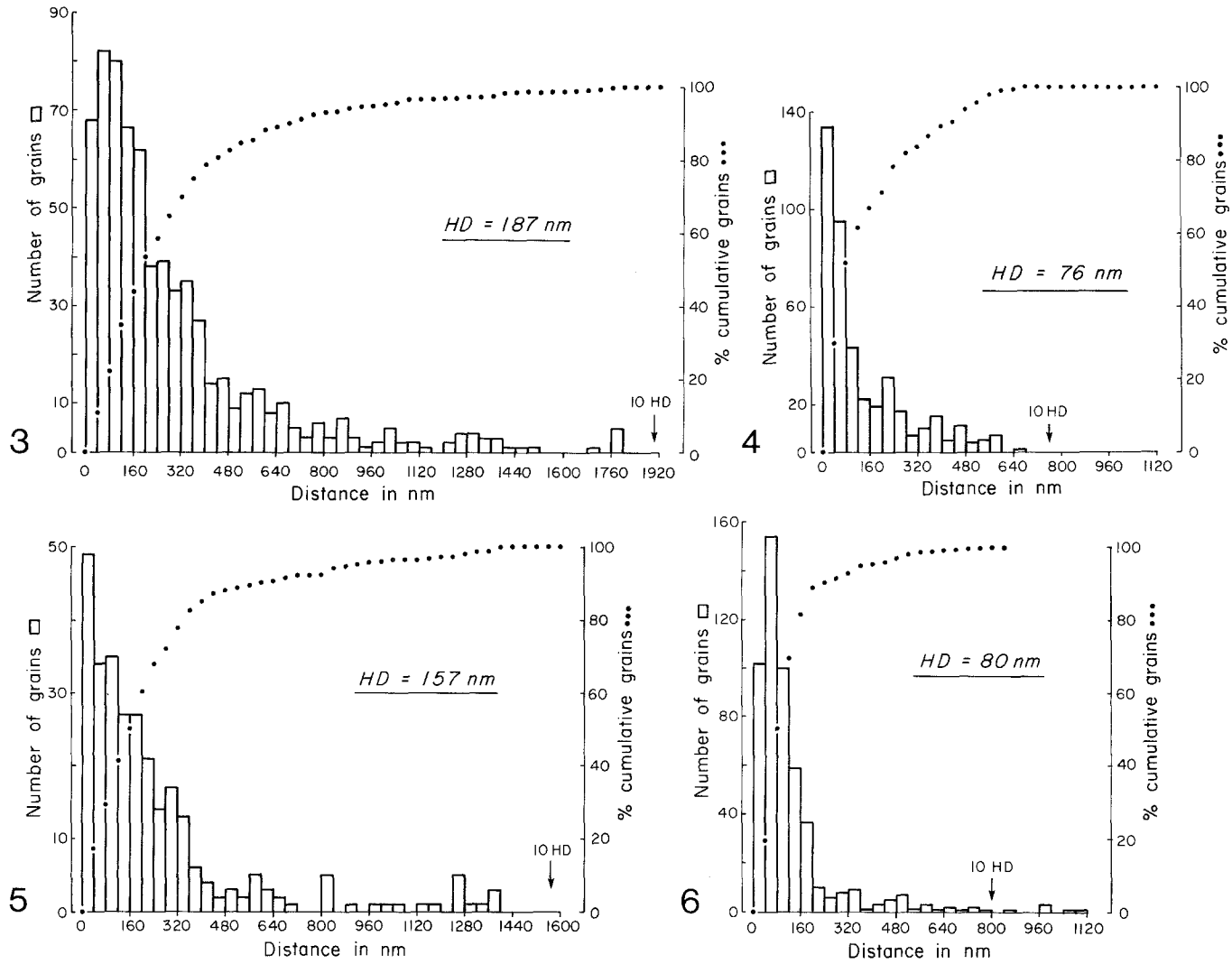
The grain counts were carried out under conditions such that the radioautographic reaction was weak enough to consist of distinctly spaced, isolated silver grains, which were either filamentous, or single spherical silver deposits or clusters of silver deposits considered as one grain. Hence, overexposure of radioautographs was avoided, since it would result either in a mass of silver deposits or a meshwork of silver filaments which cannot be allotted to individual silver grains (Kopriwa 1975).

3) Measurements, quantitation and calculations

The distance from the center of each silver grain to the center of the line source was measured to the nearest 0.05 mm with a measuring magnifier (Bausch and Lomb, USA) on micrographs printed at ×25,000 final magnification. The measured distance of 1 mm on the print was equal to 40 nm distance in the actual preparation leaving the possible error of measurements not greater than 2 nm.

The center of the large, filamentous silver grains developed with D-19b (Fig. 1) was determined by placing a transparency with concentric circles over each grain to indicate the geometric center of the grain. With SP development, silver grains consist either of a single, compact spherical silver deposit or of a cluster of two or more small silver deposits (Fig. 2) A cluster of silver deposits was interpreted as one silver grain when it fitted within the space of a silver bromide crystal, since the cluster was derived presumably from several latent images of a single exposed crystal (Kopriwa 1975). To assign a cluster to one silver grain and to determine its center, a transparency of a 3.5 mm circle (corresponding to the mean 140 nm diameter of silver bromide crystals in L4 emulsion at ×25,000 magnification) was placed over the cluster to indicate the geometric center of that grain (Fig. 2).

The distance from the line source of every silver grain was measured on both sides of the line bordered on one side by Epon and on the other side by celloidin (Figs. 1 and 2). The number of silver grains per unit of 40 nm distance from the line on the "Epon side" and the "celloidin side" was plotted on graphs. From these data, histograms of grain densities per unit distance on both sides of the line were derived. To obtain normalized grain distributions, the maximum actual grain count for each study was designated as 100%. All counts per unit distance from the line were expressed as percentage of this value. The total counts obtained for both sides of the line source were compared by X^2 test for



Figs. 3–6. Graphs showing the density distribution of silver grains around line sources labeled with either ^{3}H or ^{125}I. Data were obtained from electron microscopic radioautographs coated with Ilford L4 emulsion and developed with either D-19b or solution physical development. The left ordinate corresponds to histograms presenting the number of silver grains per unit distance from the line source. The right ordinate corresponds to the integrated grain distribution showing the cumulative percentage of silver grains in relation to the distance from the line. HD values were calculated for a cut-off distance of 10 HD

Fig. 3. Data obtained with a ^{3}H-line source and D-19b development

Fig. 4. Data obtained with a ^{3}H-line source and solution physical development. The HD value is reduced by 60% as compared to D-19b development

Fig. 5. Data obtained with a ^{125}I-line source and D-19b development

Fig. 6. Data obtained with a ^{125}I-line source and solution physical development. The HD value is 50% lower than that obtained with D-19b development

2 sets of observed values. Grain distributions on either side of the line were not statistically different ($P<0.05$). Accordingly, grain counts per unit distance from each side of the line were coupled to determine one-sided displacements. Results were tabulated in histograms presenting the total number of silver grains per 40 nm unit distance from the line source. Integrated grain distributions showing the cumulative percentage of grains in relation to distance were then calculated, and HD values were determined. For the final evaluation of HD values, a "cut off" distance of 10 HD was used (Salpeter et al.1969). ("Cut off" distance is the distance from the line source beyond which silver grains were excluded from the counts.)

Results

Histograms of normalized grain distribution on the "Epon side" and the "celloidin side" of the line source show a similar pattern of grain distribution on both sides of the line with the four isotope-development combinations. When data obtained with all four experimental groups were analyzed by X^2 tests, no significant difference between grain distributions over the Epon and celloidin side was found.

The effect of type of isotope and development on the grain distribution around a line source is seen on Fig. 3–6. The left ordinate presents histograms of total numbers of silver grains per unit distance from the line source; the right ordinate corresponds to the integrated grain distribution showing the cumulative percentage of grains in relation to distance. Comparison of HD values obtained with 10 HD cut-off distance shows that fine grain SP development reduces the HD value from 187 nm to 76 nm in the case of ^{3}H and from 157 nm to 80 nm in the case of ^{125}I (Table 1).

Table 1. Effects of isotope and development on Half distance (HD) values obtained with Ilford L4 emulsion

Isotope	Development	HD (nm)	Total number of grains counted
^{3}H	D-19b	187	677
^{3}H	SPD[a]	76	439
^{125}I	D-19b	157	286
^{125}I	SPD	80	512

[a] SPD = Solution physical development

The influence of the total number of grains counted on the HD value was tested using a ^{125}I-line source and SP development. When a total of 355 grains was evaluated, the HD without cut-off distance was 86 nm; whereas, when 533 grains were evaluated, the HD value without cut-off distance was 84 nm.

Discussion

Chemical development with D-19b produces filamentous silver grains, which are considerably larger than the original silver bromide crystals from which they arise during development. SP development with Agfa-Gevaert developer preceded by latensification with gold thiocyanate produces small spherical silver deposits, which occur either singly or in clusters of two or more per silver bromide crystal (Kopriwa 1975). Since staining of the gelatin matrix around the silver bromide crystals suggests that silver deposits, either occurring singly or in clusters remain localized within the perimeter of the original crystal (Kopriwa 1975), better resolution is expected with SP than D-19b development. Moreover, the small silver deposits obtained with SP development allow a better identification of cellular organelles, since they conceal much less structural detail of the underlying specimen than the large filamentous grains.

Indeed, about 50% reduction of the HD value was found after SP development with both ^{125}I and ^{3}H (Table 1). Thus, SP development provides better resolution, since silver grains appear closer to the radioactive source. The improved resolution may be attributed as expected to the small size of developed silver deposits which seem to remain within the perimeter of the original silver bromide crystal. However, another reason may be that latent images formed at greater distances from the radioactive source are not developed by SP development despite gold latensification. This hypothesis is supported by observations of EM radioautographs of rat thyroid glands fixed 30 min after injection with $Na^{125}I$. After coating with L4 emulsion and either D-19b or SP development silver grains were found predominantly over the colloid in the lumen of the thyroid follicles. However, using D-19b development, a significant albeit small proportion of silver grains was present also over the follicular cells; while SP development failed to produce any significant radioautographic reaction over the cells. It is believed by most, that at a short time after the injection, any grains observed over the peripheral follicular cells (such as seen after D-19b development), indicate scatter of emission which emanates from radioactive iodine in the central lumen, and are thus interpreted as artifact (Nadler and Chajut 1972). Fine grain SP development seems to eliminate this artifact, because, according to the hypothesis, activated silver bromide crystals located beyond a critical distance from the radioactive source are not developable with SP development, perhaps because the incident radiation is less energetic and, accordingly, the latent images contain too few silver atoms.

When the influence of the quantity of evaluated grains on HD values was examined with several isotope-development combinations, it was found, that HD values obtained from approximately 350 total grain counts were similar to those obtained from approximately 550 total grain counts. Nevertheless, to determine HD values, it is still practical to evaluate approximately 500 grains because decreasing the total grain count increases the statistical variation in measurements.

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