RESOLUTION IN ELECTRON MICROSCOPE AUTORADIOGRAPHY

III. Iodine-125, the Effect of Heavy Metal Staining, and a Reassessment of Critical Parameters

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ABSTRACT

Resolution for ^{125}I-labeled specimens under electron microscope (EM) autoradiographic conditions was assessed experimentally. With this isotope the size of the silver halide crystal was the most important resolution-limiting factor. Heavy metal staining such as is routinely used in preparing animal tissues for EM autoradiography produced an improvement in resolution of ~15–20%. For a 500–1,000-Å biological tissue section fixed with OsO_4 and stained with uranyl acetate, we obtained resolution (half distance, HD) values of ~800 ± 120 Å using Ilford L4 emulsion and 500 ± 70 Å using a Kodak NTE-type emulsion. General aspects of resolution-limiting factors and comparison with ^{3}H and ^{14}C values are discussed.

On theoretical grounds, ^{125}I is a highly favorable isotope (see Appendix A) for electron microscope (EM) autoradiography. This was early recognized by Kayes et al. (11) in 1962. However, although some calibration studies for both sensitivity and resolution have been performed using this isotope (6, 10), the resolution attainable with this isotope under varying experimental conditions has not yet been systematically assessed. In the present study we have adapted previous calibration procedures used for tritium and ^{14}C (2, 7, 20, 22) to obtain resolution values for ^{125}I as a function of section thickness, photographic emulsions, developing procedures, and heavy metal staining as employed in Em autoradiography. The results allowed us to reassess the critical parameters affecting resolution for isotopes of different energy.

MATERIALS AND METHODS

Resolution Specimens

Two resolution specimens were used. One was to test resolution in plastic specimens (density 1.1) as a function of section thickness and different emulsion-developer combinations; the second was to test the effect of increasing specimen density by the incorporation of heavy metals such as osmium and uranium, which are used in the fixation and staining of biological material for electron microscopy.

RESOLUTION USING PLASTIC TEST SPECIMENS: The calibration specimen was modeled after that described previously for tritium and ^{14}C (2, 20, 22) in which a thin film of radioactive polystyrene was sandwiched between a polymerized Epon block and a layer of nonradioactive methacrylate. (The radioactive styrene was thus never exposed to the solvents normally involved in embedding for EM autoradiography, which would

have dissolved the styrene.) When sectioned at right angles, this sandwiched specimen produced a radioactive "line source" which was uniformly labeled throughout the thickness of the specimen. Since no iodinated styrene is available, we used ^{125}I-albumin to make a ^{125}I line source, and this necessitated several technical modifications. Albumin does not adhere to the plastic blocks and is not self-supporting for sectioning without embedding. Fortunately, the albumin can be embedded since, unlike the polystyrene, it would not be dissolved in the embedding medium. Therefore, the following procedure was adopted: ^{125}I-albumin (3 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.) in phosphate buffer and diluted in water to obtain a final protein concentration of 0.25%. The albumin was applied with a medicine dropper onto a slide held horizontally and was then drained vertically. This procedure was similar to that used for making the sensitivity specimen described previously (6). Then, to render the film insoluble in water, it was exposed to paraformaldehyde vapor for 10 min. The slide, with fixed albumin layer, was washed in water to remove any residual water-soluble components. The remaining film was scored to form small squares, and their thicknesses were measured, using an incident light interferometer (Nomarski attachment for the Reichert Zetopan microscope). Thickness values of ~250 Å were obtained. Then, using 10% hydrofluoric acid as a stripping aid, the squares were stripped onto a water surface. Only those squares were used in which the thickness of all four corners was within 10% of their average value. Each square was then picked up onto the smooth surface of a small, polymerized Epon block, and reembedded in Epon 812 monomer. When the block was sectioned at right angles, we obtained a ^{125}I line source sandwiched between two layers of Epon (Fig. 1). Although all electrons start in the albumin film, most of them will travel primarily through plastic (Epon) in their path towards the emulsion. We therefore call this specimen the "plastic resolution specimen."

RESOLUTION IN FIXED AND STAINED TISSUE: The calibration specimen to test the effect of heavy metal staining on resolution was a modification of the one devised to test its effect on sensitivity (6, 17).

Dorsal root ganglia from the newt were fixed in OsO_4 (1% in phosphate buffer for 1 h) and block-stained with uranium at 60°C (12). This produces a more intense tissue staining than we usually employ in preparing EM autoradiographic specimens, and thus can provide an upper limit to the effect of metal staining on resolution in our standardized EM autoradiographic specimens. The stained tissue was then embedded in Epon and sectioned with a new diamond knife to produce a block with a smooth surface. We then used this tissue block to pick up a square of the ^{125}I-albumin film. When this was reembedded and sectioned at right angles, we again had a radioactive line source, but this time it was sandwiched between stained tissue on one side and plastic (Epon) on the other. This will be referred to as the "stained resolution specimens" (Fig. 6). Because of the higher density of the stained tissue compared to the Epon, those electrons that travel towards the emulsion through the stained tissue are subjected to greater scattering and self-absorption. The difference in the distribution of developed grains on the two sides of the line was used to assess the effect on resolution produced by the heavy metal staining.

For comparison, we used a similar tissue block to pick up a [^{3}H]styrene film which was then covered with a film of methacrylate to make a stained tritium test specimen.

Analysis

Sections were prepared for EM autoradiography by the flat substrate method of Salpeter and Bachmann (18, 19). To assess the effect of section thickness on resolution, sections ranged in thickness from 250 to 1,500 Å. Sections were placed on collodion-coated slides, and the thickness of each section was measured using an incident light interferometer. Only those sections that were within 10% of a desired thickness were used in this study.

The sections were coated first with carbon and then with a monolayer of either Ilford L4 emulsion (deep purple interference color; silver halide crystal size = ~1,200 Å) or an improved Kodak nuclear track NTE-type emulsion, Kodak NTE2 (light gold interference color; silver halide crystal size = ~500 Å). (Kodak NTE2 emulsion will soon be commercially available. It has better sensitivity, stability, and layer-forming properties than the currently available NTE emulsion but is expected to have the same resolution. A detailed description is given in reference 24. For the purpose of discussing resolution, we consider Kodak NTE and NTE2 to be synonymous.) The Ilford L4-coated autoradiographs were developed for large grains with Gold-EAS (23) or for small grains with paraphenylenediamine (4, 5). The Kodak NTE2 autoradiographs were developed with Dektol (Kodak; diluted: one of Dektol to two of water) at 25°C as previously described (19). Exposure times were calculated so as to have a negligible probability of multiple hits on a single silver halide crystal of the emulsion (see discussion in reference 23).

The analysis for resolution was the same as that already described in papers I and II of this series (20, 22). Grids with autoradiographs, chosen randomly from all the specimens, were photographed with a Philips 201 and magnified to a final magnification of 30,000. Overlapping pictures of the complete "hot line" on every grid ensured an assessment of the entire line source. The perpendicular distance from the center of each developed grain (i.e., center of smallest circle that could circumscribe the grain) to the midplane of the radioactive line was measured. Initially, grains up to a "cut-off distance" of 1 μm from the line were included in the tabulation. (An equal area per unit distance from the line was tabulated for the entire 1 μm so that the grain counts

could easily be converted to grain density; i.e., grains per unit area at any distance.)

The grain density was then plotted with distance from the line as in paper I (20) either as a density distribution (i.e., grains per unit area) or in integrated form by consecutively adding the number of grains. In addition to describing the full grain distribution, we also determined a half distance (HD) value for the different experimental conditions. HD was introduced as a measure of resolution by Bachmann et al. (2, 20), and is the distance from the line source that contains 50% of the total grains accumulated within a cut-off distance of 10 HD. The HD value is thus obtained experimentally in several stages: first for an arbitrary cut-off distance chosen to be large relative to the expected HD (1 μm in the present study), then for a cut-off distance equal to 10 times the first HD. If the new HD is considerably larger than the initial HD, a third HD is obtained with a cut-off distance 10 times the second HD, etc., until the values converge.

RESULTS

Fig. 1 (*a–c*) shows sample EM autoradiographs obtained from the plastic resolution specimen, i.e., without heavy metal staining, for different emulsion developer combinations; and Fig. 2 gives a sample experimental grain density histogram in angstrom units obtained from this specimen. Ta-

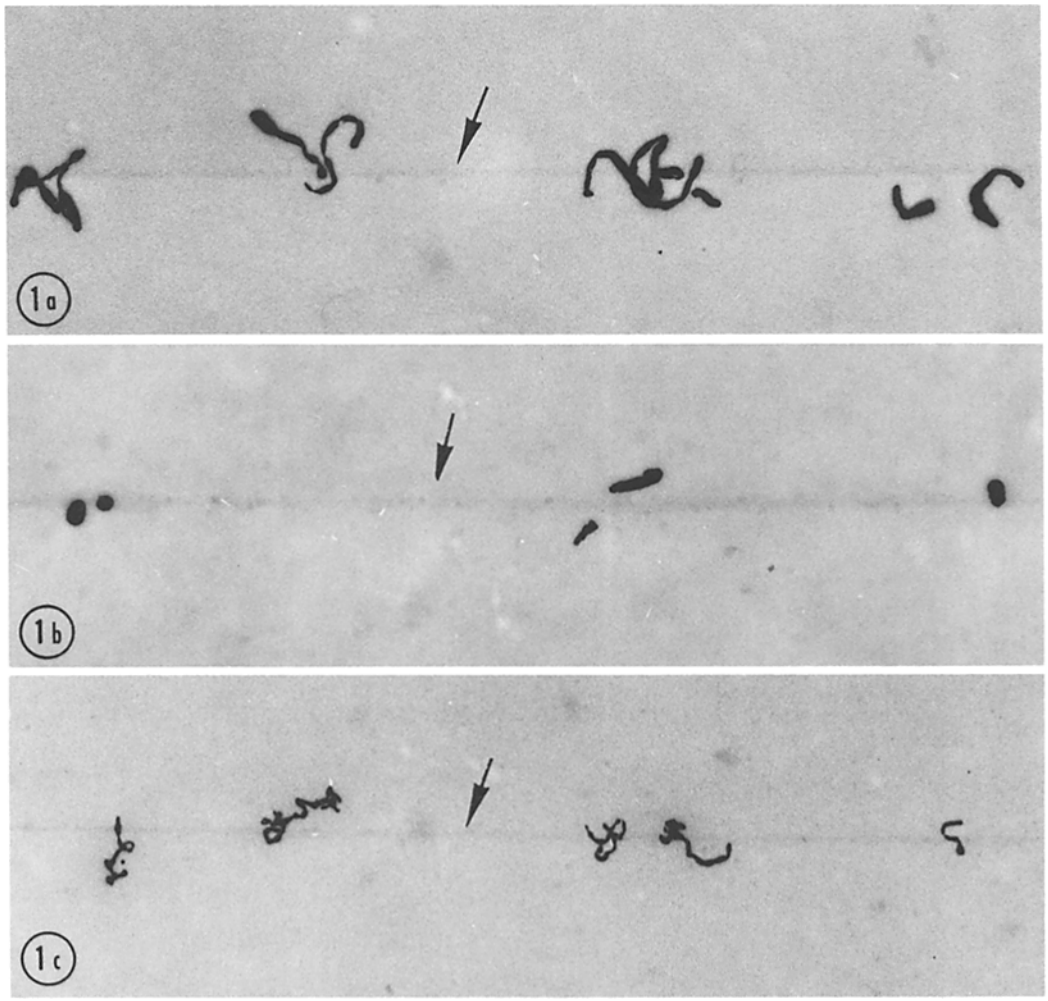

FIGURE 1 Sample EM autoradiographs obtained from plastic resolution specimens, i.e., [^{125}I]albumin film embedded in Epon and thus forming ^{125}I line source (arrows) sandwiched between layers of plastic. 1,000 Å-sections and (*a*) Ilford L4 emulsion, Gold-EAS development; (*b*) Ilford L4 emulsion, paraphenylenediamine development; (*c*) Kodak NTE2 emulsion, Dektol development. × 38,500.

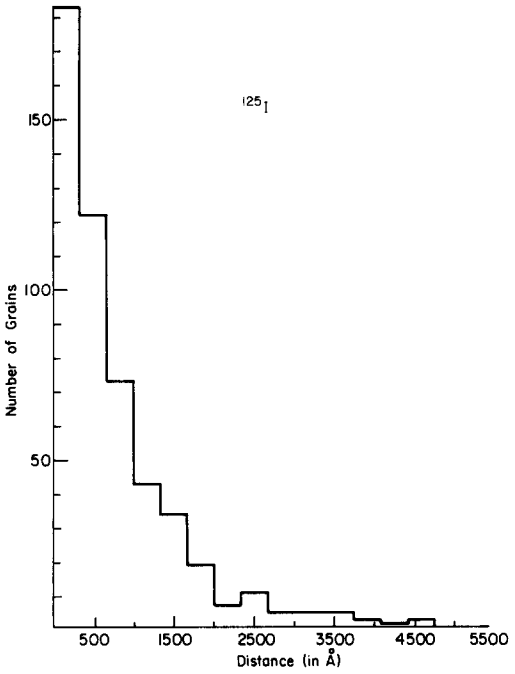

FIGURE 2 Sample experimental grain density histogram for autoradiographs from plastic specimens as in Fig. 1. A 1,000-Å section and Kodak NTE2 emulsion.

ble I gives experimental HD values obtained with this plastic resolution specimen for different section thicknesses, and different emulsion developer combinations.

Table I shows that, for each emulsion used, the ^{125}I resolution is better than that for tritium (2, 20). Furthermore, for ^{125}I the resolution appears little affected by section thickness and developed grain size. The most important single factor affecting resolution with this isotope is the emulsion and therefore the size of the silver halide crystal. (The silver halide crystal size for Ilford L4 emulsion is 1,200 Å and for the Kodak NTE2 emulsion is ~500 Å.) In the Discussion, these findings are related to our general understanding of the factors that influence resolution in EM autoradiography. Since for each single emulsion the resolution for the different specimens appeared to be within statistical fluctuation, we consider their average values as a reasonable representation for that emulsion, applicable to all of the plastic specimens tested (i.e., HD = 900 ± 90 Å for Ilford L4 emulsion and 550 ± 40 Å for the Kodak NTE2 emulsion).

Once an HD value was obtained for each specimen, the grain density histograms were replotted in distance units of angstrom/HD. A composite grain density histogram was constructed for all the specimens combined (Fig. 3). Again, as for ^{3}H and ^{14}C, once normalized in distance units of HD, all the individual specimens fall on the same general curve. This allowed us to obtain a "universal curve" as previously done for the tritium. The tritium universal curve had given a reasonable fit to the data for ^{14}C (22) and also for ^{55}Fe (15), except for a discrepancy in the tail of the distribution (the higher energy isotope ^{14}C having the longer tail). We therefore first tried to fit the tritium curve to the ^{125}I composite histogram (broken line curve in Fig. 3). The ^{125}I histogram deviates from the tritium universal curve in the opposite direction from the ^{14}C distribution, i.e., by having a shorter tail. The best fit universal curve (solid curve, Fig. 3) was obtained by modifying the tritium distribution using curve-fitting parameters given in Appendix C, and normalized to have an area equal to that of the experimental density distribution. Comparisons of experimental density distributions for line sources labeled with ^{125}I, tritium, and ^{14}C for 1,000-Å sections and Ilford L4 emulsion are presented in Fig. 4. The universal integrated form for a ^{125}I line source is graphed in Fig. 5*a*. Integrated universal curves for point sources for all three isotopes are given in Fig. 5*b*.

The effect of heavy metal staining on the resolution was examined using the stained resolution specimen as described in Methods. A sample autoradiograph is given in Fig. 6. In this specimen, electrons leave the source travel either through

TABLE I

*Experimental Values for HD for ^{125}I**

Emulsion	Section thickness‡	Developer§	Number of grains	HD
	Å			Å
Ilford L4	500	Gold-EAS	556	920
	1,000	"	982	840
	1,500	"	398	1,020
	500	Paraphenyl-enediamine	358	820
	1,350	Paraphenyl-enediamine	324	860
				av. 890 ± 90
Kodak NTE2	300	Dektol	224	580
	500	"	329	520
	1,000	"	387	550
				av. 550 ± 40

* Plastic resolution specimens, no heavy metal staining.
‡ All section thicknesses were measured by interferometry. Only those sections whose thickness was within 10% of stated value were used.
§ For size and appearance of developed grains, see Fig. 1.

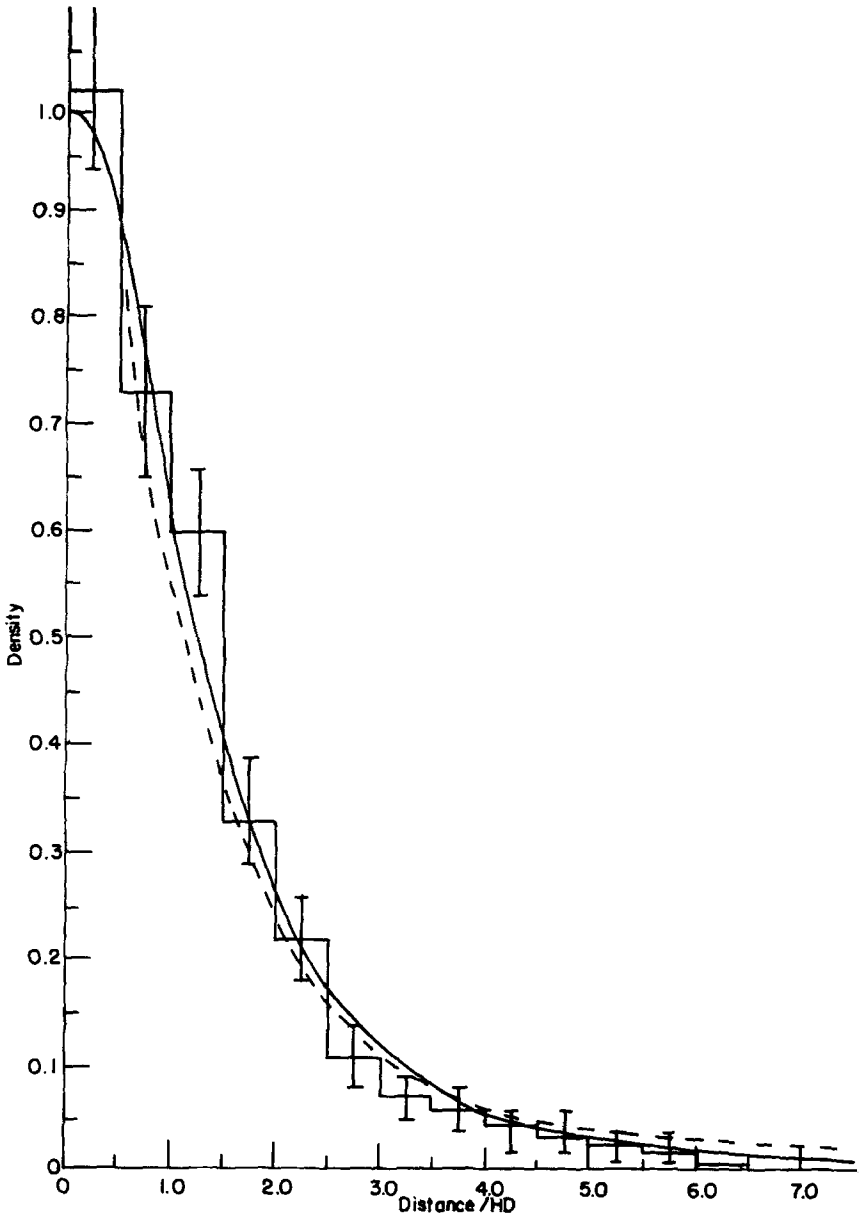

FIGURE 3 Composite histogram for all specimens including different section thickness, and emulsion-developer combinations plotted in distance units of HD. Best fit universal curve for ^{125}I line source obtained as described in Appendix C is given as solid curve. The universal curve for tritium is given as broken-line for comparison.

stained tissue or through the plastic on their way towards the emulsion (except for the few that travel straight up through the hot line itself). HD values were obtained separately for the two sides of the line source for two different specimens, both with 1,000-Å thick sections but one coated with Ilford L4 and the other with the Kodak NTE2 emulsion. We found that in both cases the stained tissue side had an HD value ~20% lower than that over the plastic side. (For the Ilford L4 specimen, the HD values for the plastic side and stained tissue side were 865 and 700 Å, respectively, and for the Kodak NTE2 emulsion they were 560 and 480 Å, respectively.) We see that

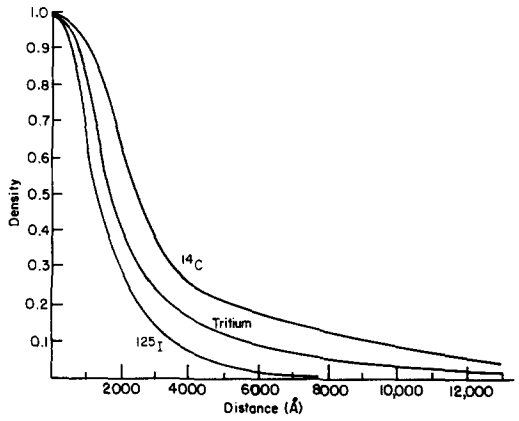

FIGURE 4 Comparison of density distribution for ^{14}C, tritium, and ^{125}I. Curves represent the best fit to grain distribution histograms from line sources in 1,000 Å-sections coated with Ilford L4 emulsion.

the values over the plastic side were within the experimental error of those obtained earlier with the plastic specimen reported in Table I and are thus a confirmation of those values. Similar studies with the tritium stained resolution specimen showed no significant difference between plastic and stained tissue side.

These results are consistent with those we have reported for the effect of uranium staining on self-absorption and sensitivity. Here, too, OsO_4 fixation and 60°C uranium staining (12) had a negligible effect on EM autoradiographic sensitivity when tritium was the source, and produced a 15% decrease in sensitivity when ^{125}I was the source (6, 17). Our results seem to be at variance with those reported by Gupta et al. (9) where a different type of specimen was involved.

In order to see an upper limit to the effect of heavy metal staining, the "stained resolution specimen" has a more intense heavy metal staining than that routinely used by us for EM autoradiographic specimens where block and section staining is done with 1% uranyl acetate at room temperature (19). The greater intensity of staining of the "stained test specimen" relative to our standard EM autoradiographic specimens is confirmed in reference 17. We therefore conclude that the most reasonable HD value for ^{125}I-labeled specimens prepared by our standard procedure falls between that for the heavily stained test tissue and that for the plastic specimen given in Table I. Reasonable intermediate HD values are: 800 ± 120 Å for the Ilford L4-coated specimen; and ~500 ± 70 Å for the Kodak NTE-coated specimen.

More heavily stained tissue would of course give a gain in resolution, but such specimens would need to be recalibrated for sensitivity and would also run the risk of differential staining of different organelles within a cell and thus introduce systematic errors that are difficult to control.

DISCUSSION

Reassessment of Critical Parameters Affecting EM Autoradiography

Early theoretical discussions on resolution in EM autoradiography (1, 4, 8, 13, 16) all basically agreed that resolution would be affected by specimen geometry (section and emulsion thickness and density, and energy of the isotope) and by the photographic process (sizes of emulsion silver halide crystals and developed grain size). What was not so obvious was the extent to which each of these factors would influence resolution both in EM autoradiography and in light autoradiography using semithin sections. Bachmann and Salpeter (1) pointed out that since resolution-limiting factors would increase as the square root of the sum of their squares, their relative importance would differ for different specimens, with the largest limiting factor being dominant.

Over the past 10 yr, these parameters have been assessed experimentally. The extent to which specimen thickness affects resolution depends on the energy of the isotope, being a more important factor for the higher energy isotopes. This is seen in comparing ^{14}C, ^{3}H, and ^{125}I. For instance, for ^{14}C, both emulsion and section thickness, even if >1-μm thick, have a considerable effect on resolution (21, 22). For ^{3}H, the emulsion thickness beyond ~1,700 Å (monolayer of Ilford L4) and section thickness beyond ~0.5 μm no longer affect resolution (19, 20), For ^{125}I, as the present study shows, variations in section thickness, even within 500 Å-1,000 Å, has a minimal effect on resolution. In addition, increasing section density by metal staining in this thickness range already tends to improve resolution for ^{125}I, whereas no such effect is yet seen for tritium-labeled sections. (For a more detailed discussion, see Appendix B.) Fig. 7 gives HD values for various isotopes as a function of section thickness, and Table II gives them for 1,000-Å sections coated with different emulsion layers. These data are derived from an

accumulation of experimental results (20–22; and the present study).

When considering the photographic factors, we again note that the largest limiting factor dominates the experimental resolution value. Thus, for ^{14}C, where specimen thickness has a large effect, varying the silver halide crystal size from ~1,300 Å (Ilford L4) to ~500 Å (Kodak NTE) makes no significant difference in HD for similar section and emulsion thickness (Table II; cf. 1,000-Å section with either a monolayer of Ilford L4 or a double layer of Kodak NTE). For tritium, where specimen thickness is less important, silver halide crystal size is already significant. The difference in HD between a monolayer of Ilford L4 and an equal thickness (double) of Kodak NTE is ~20% and for ^{125}I (present study) the silver halide size becomes dominant.

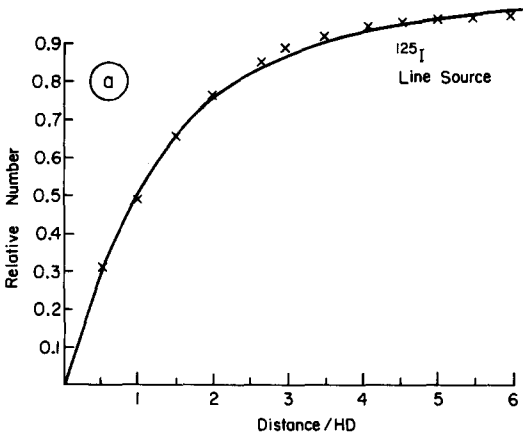

FIGURE 5*a* Integrated distribution for a ^{125}I line source. Relative number gives the fraction of developed grains with distance from the line; x's mark experimental values. (Distance in units of HD.)

The question of the effect of developed grain size on resolution has been of interest to EM autoradiographers for a long time. Because the developed grain size is the most obvious feature in an autoradiograph, the tendency has been to equate its size with resolution. On theoretical grounds, we argued (1) that resolution should be affected somewhat less by the developed grain size than by the size of the silver halide crystal, even under the least favorable assumption of silver grain development, i.e., that the developed grain grows randomly away from the latent image.

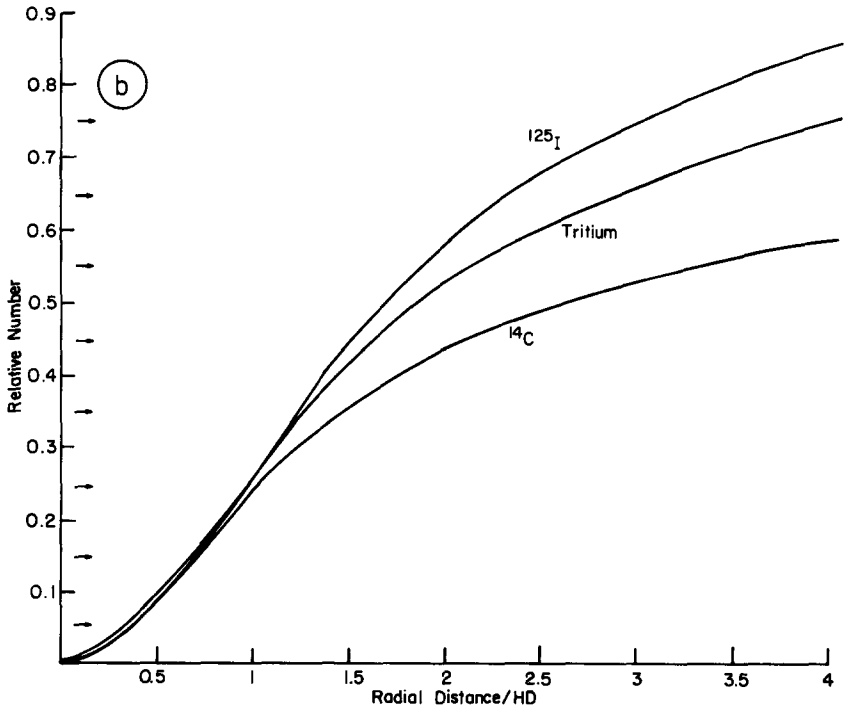

FIGURE 5*b* Comparison of integrated distributions for ^{14}C, ^{3}H, and ^{125}I point sources. (x's give experimental values; distance in units of HD.)

FIGURE 6 EM autoradiograph of resolution specimen designed to test the effect of heavy metal staining. Epon-embedded tissue block fixed with OsO_4 and stained with uranyl acetate at 60°C is on one side (bottom), and Epon 812 is on the other side (top) of the ^{125}I-albumin film or line source (arrow). Most electrons travel in part either through stained tissues or Epon to reach the emulsion. HD values were obtained separately for the two sides of the line. A 1,000-Å section and Ilford L4 emulsion. × 30,000.

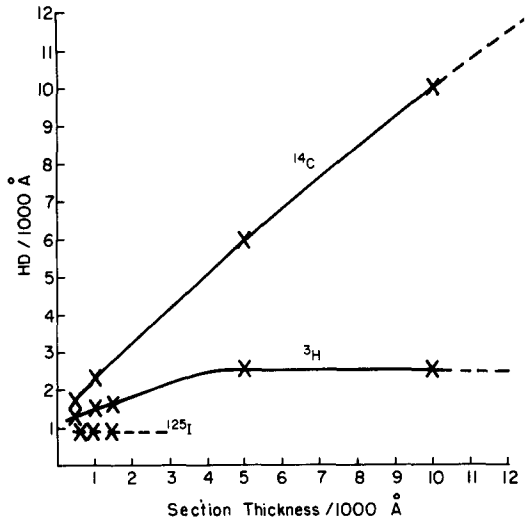

FIGURE 7 Resolution (HD) as a function of section thickness for autoradiographs coated with a monolayer of Ilford L4 emulsion. ^{125}I-autoradiographic values include heavy metal staining as routinely employed in preparing EM autoradiographic specimens, while values for ^{14}C and ^{3}H do not. (See text.) Of interest to high voltage EM autoradiography, heavy metal staining is not expected to affect these ^{14}C-values, but may cause the ^{3}H-curve to level off slightly below the 2,500 Å given here.

However, with ^{125}I, where for EM autoradiography the photographic error is the limiting factor in resolution, one would already expect to see an appreciable effect of grain size on resolution if the model of random growth of the developed grain from a latent image is valid. Table I shows this not to be the case. Gold-EAS (developed grain size ~3,000 Å) and paraphenylenediamine (developed grain size ~700 Å) give very similar HD values. It appears therefore that the center of a large developed grain is not much farther from the latent image than is that of a small developed grain, and therefore that the developed grain does *not* grow randomly from the latent image but grows more symmetrically around the silver halide crystal from which it was derived.[1] The use of fine-grained developers is in some cases still advantageous over the use of large-grained developers, since smaller grains do not obstruct the underlying fine structure and do facilitate grain counting in regions of high grain density. However, one should not assume a significant effect on resolution, and one should be aware that fine-grained developers tend to have less reproducible sensitivity.

In conclusion, we now feel able to give some practical advice to users of EM or high resolution light autoradiography regarding optimum conditions and resolution values for the three isotopes: ^{14}C (or ^{35}S), ^{3}H, and ^{125}I. For ^{14}C (or ^{35}S), section and emulsion thickness are the most important determinants of resolution, and for optimum resolution the thinnest sections and emulsion layers are advised. (HD values of ~1,800 Å can be obtained with grey sections and Ilford L4 monolayers.) The only restriction on this advice comes

[1] This conclusion is compatible with very preliminary unpublished results in which we used phosphotungstic acid to stain the gelatin of an emulsion during development and thus visualized both the growing developed grain and the silver halide crystal from which it was derived.

TABLE II

Resolution (HD) for Different Energy Isotopes in 1,000 Å Stained Sections

Isotope	Emulsion	HD/Å
^{14}C (^{35}S)	Ilford L4:	
	Double layer	2,850
	Single layer	2,300
	Kodak NTE type:*	
	Double layer	2,500
	Single layer	2,000
^{3}H	Ilford L4:	
	Single or double layer	1,500
	Kodak NTE type:	
	Double layer	1,250
	Single layer	1,000
^{125}I	Ilford L4:	
	Double or single layer	800
	Kodak NTE type:	
	Double or single layer	500

* Resolution for Kodak NTE and that for the new NTE2 are considered synonymous.

from limitations owing to low sensitivity obtained with the high energy isotopes. Fine-grained emulsion such as Kodak NTE is hardly an advantage.

For tritium the story is more complicated. Improved resolution is still obtained with thinner sections and fine-grained emulsion (Kodak NTE2 emulsion). However, the gain in resolution (~13%), in going from a 1,000-Å (HD = ~1,500 Å) to a 500-Å section (HD = ~1,200 Å), is not so great as is the loss in grain yield which is linear with section thickness in this thickness range. A fine-grained emulsion such as a Kodak NTE monolayer gives better resolution than an Ilford L4 monolayer (by 33%), owing to both its finer grain and thinner layer, and is recommended if the tissue has enough radioactivity, especially since the improved NTE2 emulsion has a twofold higher sensitivity than the standard NTE (24). The situation becomes unequivocal again in the semithin section range where going from a 0.5-μm section to a 1.0-μm section produces no loss in resolution (Fig. 7) while still giving a 33% increase in grain yield (see Table II in reference 21). Going beyond a 1.0-μm section causes neither a significant loss in resolution nor increase in grain yield.

For an ^{125}I-labeled tissue, there is no advantage to going to a section thickness lower than 1,000 Å. Doing so entails a loss in grain yield and no significant gain in resolution. A fine-grained emulsion like Kodak NTE2 gains 38% in resolution and is recommended since specific activities with ^{125}I are high as is sensitivity (6). As shown in this study, resolution of ~500 Å is thereby attainable – the best yet for EM autoradiography.

APPENDIX A

^{125}I is not a beta emitter but decays by electron capture from both the K and L atomic shells, followed by emission of nuclear gamma rays. These captures are accompanied by the emission of Auger electrons or X rays from the electron shell. The gamma ray may escape or may result in the further ejection of Auger electrons or electrons of internal conversion (14). In total, per 100 nuclear decays there are emitted: 36 high energy (22–34.2 keV), and 128 low energy (2.77–3.6 keV) internal conversion and Auger electrons, as well as 7 gamma rays (35.4 keV), and 143 X rays at 24.7–35.4 keV.

Since specific activity is expressed in terms of nuclear events, ^{125}I provides an amplification factor emitting 1.64 electrons and 1.5 photons per nuclear event. In addition to this amplification factor, ^{125}I also differs from the beta emitters, like tritium or ^{14}C, by having discrete energy peaks rather than a continuous energy spectrum. The majority of the electrons (80%) are in the low-energy range (approximately 3–4 keV) and thus are expected to have both high sensitivity and good resolution.

APPENDIX B

Figs. 8*a* and *b* give a qualitative argument compatible with the discussion on the effect of geometric factors on resolution. The role played by section thickness and staining comes from the interactions between the electron leaving its source and the material through which it travels on the way to the emulsion. In Figs. 8*a* and *b*, we consider a radioactive source near the bottom of a tissue section of thickness either t_1 or t_2. Electrons are emitted in all directions, but we consider only the half emitted in the direction towards the emulsion: one such electron, if it travels in a straight path (Fig. 8*a*), will hit the emulsion at either g_1 or g_2, depending on the thickness of the section. Since g_2 is at a greater horizontal distance (x_2) from the source than is g_1, (x_1), it is clear that resolution is poorer with a thicker section. However, *if* the electron is of such low energy that its range in the tissue is less than the distance to g_2, then this electron will be absorbed and never reach the emulsion. Consequently, resolution deteriorates less as a section becomes thicker relative to the range of electrons as is illustrated on the left side of Fig. 8*a*. (Any electrons not reaching the emulsion cannot be registered as a developed grain, and thus the grain yield or number of developed grains obtained ceases to be linear with section thickness. Since sensitivity is grains/decays, sensitivity should go down in this thickness range.)

Electrons will not travel forever in a straight line path, but they will be scattered and change course and thus

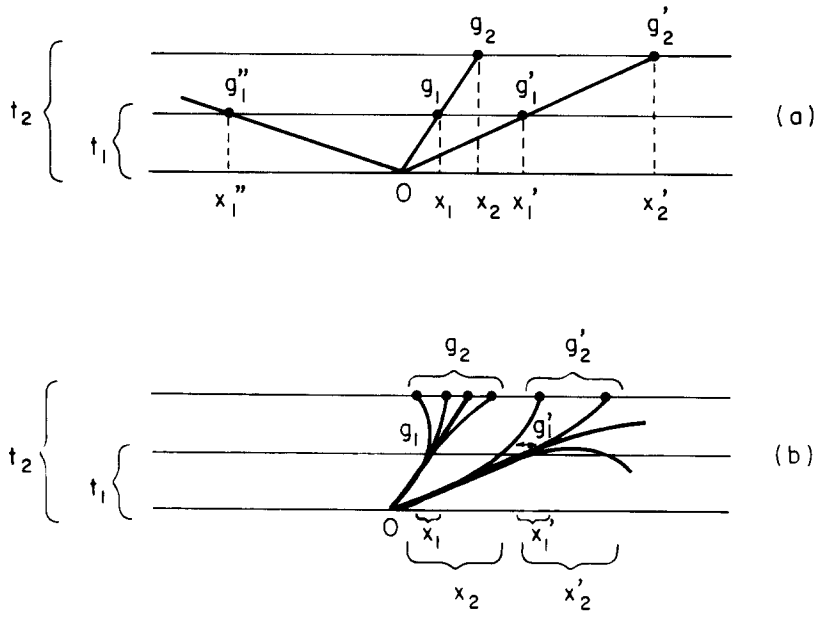

FIGURE 8 Geometric factors in resolution: schematic illustration of the effect of section thickness without scattering (*a*) and with scattering (*b*) on resolution and grain yield. A source (*O*) at the bottom of a section of thickness either t_1 or t_2 emits electrons which form developed grains g_1 or g_2 in the overlying emulsion at horizontal distances (x_1 or x_2) from the source: x_1' and x_2' are distances equivalent to x_1 and x_2 but for an electron radiating from the source at a more slanting angle. Scattering is seen to improve resolution since on the average the horizontal distances are shorter in (*b*) which includes scattering than in (*a*). These differences are most marked with thicker sections and more slanting paths (e.g. x'_2).

deviate from the straight line geometry of Fig. 8*a*. An electron can have its direction of travel changed either towards the emulsion or away from it (trajectories in Fig. 8*b*). The fraction of the electrons deviated away from the emulsion, again, will not affect resolution because they will never see the emulsion. However, an electron deflected towards the emulsion can produce a developed grain in the emulsion closer to the source than if it had continued on a straight path. Scattering is always greatest at the end of the path and increases with increasing specimen density. Thus, scattering tends to improve the resolution and counteract the detrimental effect of increasing section thickness. Therefore, as section thickness is increased, there is, in principle, a section thickness range where electrons are considerably scattered yet are still hitting the emulsion. Within this range, sensitivity is not yet significantly decreased but resolution no longer continues to significantly worsen. (Average values of x_2 in Fig. 8*b* are smaller than x_2 in Fig. 8*a*.) For the same reasons, if a section is of a given critical thickness relative to an isotope, the increasing scattering by metal staining will produce an improvement in resolution. It is difficult to predict these critical thicknesses accurately, but it clearly must depend on the energy (thus range) of the electron and on the density of the material in which the electron travels (the higher the density, the thinner a specimen will be as it approaches the critical thickness).

APPENDIX C
(*Mathematical*)

The most basic aspect of resolution in an autoradiographic technique is the grain density function $f_p(r)$ at distance r from a point source. Theoretical models (discussed, for instance, by Bachmann and Salpeter [1] and Bachmann et al. [2]) cannot be made very accurate, and detailed experiments are available only for the grain density function $f_l(x)$ at distance x from a *line* source (described in paper I [20] for tritium sources, in paper II [22] for ^{14}C, and in the present paper for ^{125}I). We discuss below how to infer the function f_p from an experimentally determined function f_l by first fitting an analytical expression to f_l. Once f_p is known, predictions can be made for other source distributions (which can be represented as sums over many point sources) by generating "universal curves" for simple source geometries (20) or by computer simulation for complex geometries (3).

Let $f_l(x)$ be the grain density function at normal (shortest) distance x from a uniformly labeled straight line source, normalized to unity at the origin so that $f_l(0) = 1$. On the other hand, let $f_p(r)$ be the grain density function

at radial distance r from a point source. The general relation between f_l and f_p is then

$$f_l(x) = \left[\int_0^\infty f_p(y)dy\right]^{-1} \int_0^\infty f_p([x^2 + y^2]^{1/2})dy. \quad (1)$$

We shall also need the "integrated curves" for both point sources and for line sources, normalized to unity at infinity

$$F_p(r) = \left[\int_0^\infty f_p(r')r'dr'\right]^{-1} \int_0^r f_p(r')r'dr', \quad (2)$$

and

$$F_l(x) = \left[\int_0^\infty f_l(x')dx'\right]^{-1} \int_0^x f_l(x')dx'. \quad (3)$$

The "relative number" $F_p(r)$ for a point source is the fraction of all developed grains that fall within a circle of radius r surrounding the source; the "relative number" $F_l(x)$ for a line source is the fraction of all developed grains that fall within a band at normal distance $-x$ to $+x$ from the line source.

As discussed in papers I and II (20, 22), the simplest kind of theoretical model leads to functions of the form

$$f_p(r) = \frac{1}{[1 + (r/d)^2]^{3/2}}, f_l(x) = \frac{1}{[1 + (x/d)^2]}, \quad (4)$$

where d is a constant for a given experimental arrangement (but depends on section thickness and on the emulsion). From equation 4 it follows that $F_l(x = d) = 0.5$, i.e., half of all the developed grains for a line source fall within normal distance d of the source. This simple model was based on a number of assumptions and approximations, such as neglecting scattering and absorption in the biological section but assuming rapid scattering and absorption in the emulsion. These approximations are reasonably good for a radioactive source of intermediate energy such as tritium; but, for a high energy source, such as ^{14}C, scattering is slow even in the emulsion and, for a low energy source, such as ^{125}I, electrons traveling at large slant angles can be scattered and absorbed even in the section. These inaccuracies in the simple model mainly affect the "tail of the distribution," i.e., f_p and f_l fall off less rapidly at large distances than indicated by equation 4 for ^{14}C, but more rapidly for ^{125}I.

Tritium sources with different emulsions and section thicknesses were discussed in paper I (20). Although the resolution varies from case to case, the grain density function f_l has an almost "universal shape" when the distance x is expressed in units of a quantity HD. The quantity HD is one convenient measure of resolution and is essentially the normal distance from a line source inside which half the developed grains fall (HD would equal d if equation 4 were valid). In practice, it is convenient to cut off the tail of the grain distribution function at some large but finite value when defining a measure of resolution. As a cut-off distance, we adopt 10 times HD itself. Mathematically, our definition of HD is expressed by the relation

$$F_l(x = \text{HD}) = 0.5\ F_l(x = 10\ \text{HD}). \quad (5)$$

We shall call $Z \equiv x/\text{HD}$ the normalized normal distance from a line source and $R \equiv r/\text{HD}$ the normalized radial distance from a point source. (Since HD was defined for a line source, the fraction of developed grains around a point source that falls within a circle of normalized radius $R = 1$ is not one-half, e.g., for tritium and ^{125}I the fraction is ~0.3 and for ^{14}C it is ~0.25. We call the radius around a point source containing half the grains HR.) Our cut-off is applied only in defining HD and not to the integrals in equations 2 and 3.

The experimental curves for the grain density function f_l for line sources with tritium, ^{14}C, and ^{125}I can be fit quite well by an analytic expression with three adjustable parameters α, β, and C. This expression is

$$f_l(x) = \frac{\alpha^2\beta^2}{\beta^2 + a\,\alpha^2}\left[\frac{1}{\alpha^2 + Z^2} + \frac{C}{\beta^2 + Z^2}\right]. \quad (6)$$

Equation 4 is a special case of equation (6) with $\alpha = 1$ and $C = 0$. The second term in equation 4 mainly affects the tail of the density function (especially for $Z \gtrsim \beta$) with positive a increasing the tail and negative a decreasing it.

An explicit analytic form like equation 6 has the advantage that it is uniquely related to another analytic form for the density function f_p for a point source,

$$f_p(r) = \frac{\alpha^3\beta^3}{\beta^3 + a\,\alpha^3}\left[\frac{1}{(\alpha^2 + R^2)^{3/2}} + \frac{C}{(\beta^2 + R^2)^{3/2}}\right]. \quad (7)$$

The "integrated curve" or "relative number" F_l and F_p (for a line source and point source, respectively) can then be obtained from equations 2 and 3. This gives

$$F_l(Z) = \frac{2}{\pi}\left(\frac{\beta}{\beta + a\alpha}\right) \left[\tan^{-1}\left(\frac{Z}{\alpha}\right) + \frac{C\alpha}{\beta}\tan^{-1}\left(\frac{Z}{\beta}\right)\right], \quad (8)$$

$$F_p(r) = 1 - \left(\frac{\alpha\beta}{\beta + a\alpha}\right) \left[\frac{1}{(\alpha^2 + R^2)^{1/2}} + \frac{C}{(\beta^2 + R^2)^{1/2}}\right], \quad (9)$$

with $Z = x/\text{HD}$ and $R = r/\text{HD}$ as usual.

Although there are three adjustable parameters α, β, and C in equation 6, only two are genuinely independent since equation 5 must also be satisfied. Explicitly, this requirement is

$$\frac{\alpha}{\beta}\left[\tan^{-1}\left(\frac{10}{\beta}\right) - 2\tan^{-1}\left(\frac{1}{\beta}\right)\right] = 2\tan^{-1}\left(\frac{1}{\alpha}\right) - \tan^{-1}\left(\frac{10}{\alpha}\right), \quad (10)$$

and equation 10 can be used to specify the value of C once the values of α and β have been chosen. One can choose α and β by requiring the analytic function f_t to fit the experimental f_t at two different values, Z_1 and Z_2 of Z (not counting $Z = 0$, where $f_t = 1$, automatically). For ^{14}C, the values for α and β in Table AI are based on $Z_1 \sim 1$ or 2 and $Z_2 \sim 10$ or 20. The resulting analytic expression for f_t agrees with the experimental one within the present experimental error (3σ) for all values of Z between 0 and 20 (we have no experimental results yet for $Z > 20$). For tritium, we used $Z_1 \sim 1$ or 2 and $Z_2 \sim 5$ or 10 to obtain still a slightly better fit than the expression used in paper I (20). For ^{125}I, the most striking feature of the experimental curve for f_t is that it falls off more rapidly at large values of Z than is given by equation 4, which has the tail falling off as Z^{-2} for large Z. We therefore chose $a = -1$ for ^{125}I which assures that the two terms in equation 6 almost cancel each other for large Z, and f_t falls off approximately as Z^{-3} when $Z \gg \beta \gg 1$. Choosing the value of α to fit the experimental curve in the range $Z \sim 1$ to 2 was found to give a good fit at other values of Z as well. The values of α, β, and C, found as described above, are given in Table AI for each of the three isotopes.

TABLE AI

Values for the Three Parameters α, β, and C in Equation 6

Isotope	α	β	C
^{125}I	1.378	5.477	−1.0
^{3}H	1.035	5.00	+0.466
^{14}C	0.8944	12.25	+5.265

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