

ABSOLUTE SENSITIVITY OF ELECTRON MICROSCOPE RADIOAUTOGRAPHY

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ABSTRACT

A calibration method is described for measuring absolute radioautographic sensitivities under various experimental conditions. Sensitivities to ^3H and ^{35}S radiation, i.e. ratio of developed grains to radioactive decays in the specimen, were determined with Ilford L4 and Kodak NTE emulsions. The highest values obtained in monolayers of emulsion were $\frac{1}{8}$ for ^3H and $\frac{1}{21}$ for ^{35}S . The influence of various experimental parameters on sensitivity is described, and the possibilities for quantitative electron microscope radioautography are discussed.

Radioautography methods are employed to locate radioactive decays and to measure the amount of radioactive substances in a given specimen area. In making electron microscope radioautography a reproducible and quantitative method the main problems arise from the large number of experimental factors which influence the results of the method.

For quantitative measurements of radioactivity by means of radioautography, the thickness of the specimen, the thickness of the emulsion layer, and the sensitivity or efficiency of the method must be known. The sensitivity of the method depends primarily on the properties of the emulsion, on the developing technique, and on the type of radiation. It also is influenced, however, by absorption and scattering of the radiation in the specimen, in the emulsion, and in the supporting layers, by chemical interaction between specimen and emulsion, and by latent image fading during prolonged exposure periods.

Experience has shown that it is more difficult to obtain reproducible results with electron microscope techniques than with light microscope

techniques. With the former, the specimen and the emulsion layer are much thinner. Therefore, variation in the thickness of either, while small on an absolute scale, will introduce a large relative error. In addition, the photographic process is used near the limits of its potential in resolution and sensitivity. Both the very fine, grained emulsions and the developing techniques employed are more sensitive to small variations in experimental conditions. However, all these factors can be controlled within limits, and the results can be reproduced to the extent that quantitative data can be expected from electron microscope radioautography. In this paper a method will be described for the determination of sensitivities which allows quantitative radioautographic measurements on an absolute scale.

In radioautography, one can consider the sensitivity either of the radioautographic method or of the emulsion layer. The sensitivity of a radioautographic method can be defined as the ratio of developed grains in a radioautograph to the radioactive disintegrations which occurred in the specimen during exposure. Such a definition is

useful since in the electron microscope the individual, developed silver grains are clearly visible and can be counted. In previous publications (1, 2) the sensitivity of an emulsion layer was defined as the probability that the radiation which hits the emulsion layer is registered, and sensitivity was expressed as the ratio of developed grains to electrons hitting the emulsion layer. In light microscope radioautography, this emulsion sensitivity also is known as the grain yield. For comparison, the sensitivity of the radioautographic method is expected to be about one-half the sensitivity of the emulsion layer, since in the usual electron microscope techniques about one-half the radiation emitted from the specimen hits the emulsion.

CALIBRATION METHOD

Relative sensitivities of various emulsion-developer combinations to β -radiation have been measured by exposing emulsion layers to an electron beam of known intensity (1, 3). Such a calibration method, however, differs in many parameters from the conditions of actual radioautography and will give only an estimate of the absolute radioautographic sensitivity. For an absolute measurement, a calibration method must be used in which all the factors influence sensitivity to the same degree as in actual radioautography. One method compatible with the requirement is the use of biological material (5, 6). However, it is difficult to know the activity over defined areas in such material with sufficiently high accuracy. A simpler method would be to use sections from radioactive plastic material. However, these methods do not allow the independent study of specific interactions between section and emulsion under varying fixation and staining conditions. Furthermore, in both these cases, any errors in measuring specimen thickness and its variations over different areas will influence the calibration. The method to be described here also fulfills the requirement and allows the direct measurement of absolute sensitivities under a greater variety of experimental conditions than either of the above mentioned procedures.

In this method the well-known procedure for testing sensitivity of radiation detectors has been adapted. An extended radioactive source of known activity and of a thickness and density similar to those of an actual radioautographic specimen is pressed against a slide which is coated with an emulsion. After exposure the slide with the emulsion is separated from the calibration source and is processed, either immediately or after controlled storage periods. The number of silver grains then is determined in relation to the radioactive decay of the isotope.

Preparation of Calibrated Radioactive Source

The calibration specimen consists of a microscope slide coated with a 500–1000 Å layer of gelatin mixed with a radioactive substance. Radioactive material was added to a filtered, aqueous solution containing a known amount of gelatin. The specific activity was so high that the amount of the radioactive substance was negligible compared to the amount of gelatin.¹

Uniform layers with a low thickness gradient were obtained when the active gelatin solution was dropped on a microscope slide and dried in a vertical position. A single coating with 0.5% solution forms layers 400–500 Å thick. Thicker layers were obtained by multiple coating. When dry, each slide was placed on a drawing as shown in Fig. 1. Two parallel lines with three positions were scratched into the layer as illustrated, and the thickness at positions 1, 2, and 3 on the upper and lower lines was measured with a Normarski interferometer for incident light (Reichert). (The contrast of the interference lines was increased by placing the microscope slides on a piece of black glass to which they were coupled optically with a drop of water.) In this way an accuracy of approximately ± 30 Å was obtained. Such high accuracy cannot be obtained in measuring the thickness of ultrathin sections because their surfaces are not sufficiently smooth. With the calibration specimen even higher accuracy could be obtained with multiple beam interferometry. However, this additional effort does not seem to be necessary. For the calibration experiments described here, the only specimens used had a thickness difference of 10% or less between the upper and lower lines. By taking the mean of the two values, the thickness of the film at the marked positions between the lines was determined with about 5% accuracy.

The density of the gelatin had been determined by the immersion method. Since the added radioactivity per gram of gelatin was known, the activity of the film at the calibrated areas could be calculated. The activity, of course, decreases according to the half-life of the isotope and must be corrected to compensate for the age of the specimen. For tritium specimens (half-life, 12.5 yr) this change is of no importance during an exposure, but corrections must be made for the ³⁵S sources (half-life, 87 days).

Since in our laboratories an intermediate carbon layer between specimen and emulsion is used routinely in specimen preparation (2), the radioactive

¹ Methyl-³H-thymidine and ³⁵S-sodium sulfate with specific activities of about 1 mc per mg were used. The activity of the radioactive material was known with an accuracy of $\pm 5\%$. The solutions were obtained from the New England Nuclear Corporation, Boston, Mass.

test specimens also were vacuum coated with a 50 Å carbon layer.

Measurement of Sensitivity

So that the sensitivity of a radioautographic method with a specific emulsion can be measured, a thin film of the emulsion is formed on a microscope slide previously coated with collodion and carbon. Details of the coating procedures have been given in previous publications (2, 3). When the emulsion layer is completely dry it is placed face-to-face with the radioactive test slide, and the two slides are clamped together with alligator clips. After exposure the slides are separated and the emulsion is developed. In order that the background can be measured, an emulsion layer, coated at the same time and kept under identical conditions but not exposed to radiation, always is developed together with the exposed layer.

Obviously, in this method there is a gap between the emulsion layer and the radioactive source. Microscopic measurement and the observation of the Newton fringes between the two slides show, however, that this gap is only in the order of 10μ or less. If the slides are stored in air during exposure, the separating layer has a mass of $1.3\mu\text{g per cm}^2$; if they are stored in helium, as in the present study, this layer has a mass of $0.2\mu\text{g per cm}^2$. This corresponds to plexifilms with thicknesses of 100 and 20 Å, respectively. Even for the low-energy tritium radiation the absorption in such a thin layer is of little influence and, therefore, was ignored in our calculations.

Since emulsion thickness affects efficiency, a judgment must be made regarding the thickness and uniformity of the emulsion layer overlying the calibrated areas of the test specimen. To do this, the emulsion-coated slide is placed over the drawing of the calibration specimen (Fig. 1), and the position of the calibrated areas is marked on the emulsion with a needle. A fairly accurate determination of emulsion thickness can be made on the basis of interference colors in either of two ways. In the first, if a developer is used which does not affect interference color, the emulsion is developed, stopped in acetic acid, washed, and dried. The interference

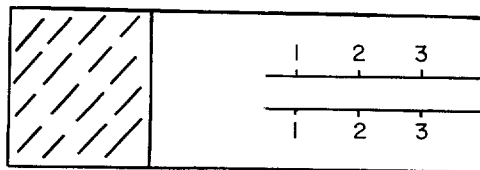


FIGURE 1 Schematic diagram of the radioactive calibration slide, showing the marking lines and the calibrated areas.

colors now are observed over the calibrated areas in white light. The emulsion layer then is fixed, and the specimen is stripped onto water, specimen grids are placed on the marked areas of the film and picked up. With the Ilford L4 emulsion the interference colors are changed by the developers and even by washing. Since the thickness calibrations were done on unwashed emulsions, the judgments on the thickness of this emulsion were made under yellow-green safe light before development. Even though interference colors appear only as density differences in the safe light, a meaningful judgment can be made. This is possible because coating procedures have been standardized to produce films which vary in thickness by less than a factor of two (2). One thus must judge only among a few adjacent interference colors. We found that an experienced observer is able to judge film thickness within better than 200 Å at the boundary between two interference colors in the safe light.²

Calculating Sensitivity Values

Electron micrographs of the processed emulsion layer are taken from a number of randomly selected grid openings, and an average value for developed grains per unit area of emulsion is obtained. The density of background grains from the unexposed control slide is subtracted from this value. The radioactive decays in the congruent area of the test specimen are calculated by multiplying the specific activity of the gelatin layer (activity per unit volume) by its thickness and by the exposure time. The ratio of the developed grains to the radioactive decays during the exposure period gives the absolute efficiency of the radioautographic method.

EXPERIMENTAL CONDITIONS AND RESULTS

The calibration method was applied to determine the radioautographic sensitivities for tritium and ³⁵S radiation with Ilford L4 and Kodak NTE emulsions. Kodak NTE emulsion was used as presently available commercially, after removing the excess gelatin by centrifugation.³ Several batches of emulsion obtained

² Interference colors, as well as interferometry, give mean values for the thickness of the emulsion layers. Therefore, if the silver halide crystals in the layers are not closely packed, values for thicknesses can be obtained which are smaller than the diameters of the silver halide crystals. Such "thicknesses" are, however, still meaningful as far as sensitivity is concerned.

³ In previous studies (1, 3) it was found that monolayers both of Ilford L4, developed with Microdol-X, and of centrifuged Kodak NTE, developed with Dektol, had the same sensitivity of 1:12 when irradiated with 10 keV electrons. Repeating these experi-

TABLE I
Sensitivities or Efficiencies of Radioautographic Methods for ^3H and ^{35}S

Emulsion	Developer	Emulsion thickness	Sensitivities	
			^3H	^{35}S
		A		
Ilford L4	Microdol X (3 min, 24°C)	1400 (Monolayer)	$\frac{1}{10}$	$\frac{1}{21}$
Kodak NTE	Dektol (2 min, 24°C)	700 (Monolayer)	$\frac{1}{23}$	$\frac{1}{70}$
		1400	$\frac{1}{12}$	$\frac{1}{36}$
Gold latensification-Elon ascorbic acid (3, 13)		700	$\frac{1}{8}$	

The sensitivities (mean values) are given as the ratios of developed grains to radioactive decays in the specimen. The average deviation of values obtained on different occasions was about $\pm 20\%$.

over a period of 1 yr were used but no consistent difference in sensitivities was noticed from batch to batch. The following developers were used: for the Ilford L4 emulsion, Microdol X (3 min, 24°C); for the Kodak NTE, Dektol (diluted 1:2, 1 or 2 min, 24°C), or Elon-ascorbic acid, preceded by gold latensification (13). Details on the developing procedures have been given in the previous publications. The experiments were performed under the simplest conditions, which eliminated possibly adverse affects of contact between section and emulsion and of latent image fading. Therefore, the emulsions were exposed for 1-8 days, separated, and developed immediately. During exposure all specimens were kept in helium at room temperature. Radiation doses from 4 to 15 electrons per μ^2 were used. The thickness of the radioactive gelatin layer in the test specimen was between 800 and 1000 A. The sensitivities obtained under these conditions are listed in Table I.

In monolayers of Kodak NTE developed with Elon-ascorbic acid after gold latensification and in those of Ilford L4 emulsion developed by Microdol X, the sensitivities to tritium were found to be $\frac{1}{8}$ and $\frac{1}{10}$, respectively, that is, one developed grain in the radioautograph indicated, on the average, 8 or 10 tritium decays in the specimen.

In the Ilford L4 layer the sensitivity for ^{35}S radiation is about two times lower than the sensitivity for tritium radiation, and in the Kodak NTE it is about three times lower. Because of the

ments, we found that the Kodak NTE emulsion as currently supplied showed a grain yield which was lower by about a factor of two. Recently some experimental fine grained Kodak emulsions had, again, a higher sensitivity. This shows that this extremely fine grained emulsion is at present not at its optimum sensitivity. It should therefore be rechecked frequently.

similar energy spectra of their radiations, the sensitivity for ^{14}C radioautography should be about the same as that for ^{35}S .

Table I also shows that a 100% increase in the emulsion thickness of Kodak NTE, from 700 to 1400 A, increases the sensitivity by the same factor for both tritium and ^{35}S radiation. We found, however, that in these, thicker layers (1400 A) gold latensification did not increase sensitivity relative to Dektol development. Slow penetration of the very dilute gold solution into the lower layer of emulsion might cause this failure to increase sensitivity. Proper modifications of the procedure must be worked out for thicker layers. Doubling the emulsion thickness in the Ilford L4 emulsion to 2700 A increased the sensitivity by only about 60%. From the sensitivity value, the exposure time, and the half-life of the isotope used (or the specific activity of the labeled compound), one can calculate the number of radioactive atoms (or the number of molecules of the labeled compound) which yield, on the average, one developed grain. For instance, if the exposure time is 4 wk, and if a monolayer of Kodak NTE is developed with gold-ascorbic acid, one developed grain in the radioautograph will indicate an average of 1800 tritium atoms in the specimen. If a monolayer of Ilford L4 is developed with Microdol X, one developed grain corresponds to about 2200 tritium atoms or 100 sulfur-35 atoms in the specimen.

DISCUSSION

Comparison with Previous Results

In our earlier experiments (1, 3), emulsion sensitivities or grain yields were obtained by irradiating emulsion layers with 10 kv electrons.

The grain yield was $\frac{1}{12}$ for monolayers of Ilford L4 (Microdol-X developed). It is interesting to compare this with the sensitivity obtained from the tritium specimen. We already have discussed the fact that, owing to the 2π geometry in actual radioautography, the grain yield values must be divided by two in order to be comparable with radioautographic sensitivity or efficiency. One then obtains from the 10 kv irradiation experiments a value of $\frac{1}{24}$, which is much lower than the sensitivity obtained from the tritium specimen. The higher sensitivity under conditions of actual radioautography with the tritium specimen is, in principle, consistent with theoretical expectation. The tritium electrons have a lower mean energy (5.5 kev versus 10 kev) and, since they are emitted from the source in all directions, they have a greater average path length in the emulsion than do electrons from a beam, all of which hit the emulsion at a right angle. A detailed discussion of these factors for light microscopic radioautography has been given by Lamerton and Harris (12).

Caro and Schnos (6) used tritium-labeled bacteria as a test source and obtained a sensitivity with monolayers of Ilford L4, of approximately $\frac{1}{5}$, which is higher than ours by a factor of two. However, since they claim only an order of magnitude for the sensitivity, and since the thickness of their emulsion layers actually was not determined, the two results do not seem to be out of line.

Specimen Thickness

Self absorption of radiation within the specimen, in principle, will cause a decrease in radioautographic sensitivity with increasing specimen thickness. However, from the energy spectrum of tritium radiation and the range of β -particles, it can be calculated that self absorption in specimens with a mass thickness of about $10 \mu\text{g}/\text{cm}^2$ will be small in relation to the over-all accuracy of the method (9). Experimental studies of tritium radiation by light microscope radioautography showed that self absorption in a 1000 Å section of biological material amounts to only 5% or less of the total radiation (7, 8). Differences of a few hundred angstroms in the thickness of the radioautographic specimen, as long as the specimen is not thicker than 1000 Å, are, therefore, of no practical influence on the sensitivity so far as self absorption is concerned. This means that constant sensitivity values can be used in electron microscope radio-

autography, where section thicknesses are 1000 Å or less, regardless of the thickness of the section. One should remember, however, that the specimen thickness must be known in order to obtain quantitative results regarding the concentration of radioactivity. We already have reported that the specimen thickness can be measured interferometrically (10) before emulsion coating (1) if a higher accuracy is desired than can be obtained by interference color judgments.

Emulsion Thickness

Sensitivity will increase with emulsion thickness as long as this thickness is less than the range of the radiation. When the range of the radiation in the emulsion and the thickness of the emulsion become equal, the sensitivity does not improve further. An increase in sensitivity due to emulsion thickness parallels a decrease in resolution. It follows that when increasing thickness no longer increases sensitivity, it cannot decrease resolution. Indeed, in light microscope radioautography often the emulsion layers used are so thick that for tritium radiation neither sensitivity nor resolution is influenced by fluctuations in the emulsion thickness. In electron microscope radioautography the emulsion layers used are thinner than the range of even the tritium electrons. Thus thickness variations do influence sensitivity. In the thickness range which interests us, e.g. 1500 Å, sensitivity is about linear with thickness.

The Dose of Radiation

The radiation per area to which the emulsion is exposed also can influence sensitivity owing to either multiple hits (more than one electron hitting one silver halide crystal) or the overlap of developed grains (more than one developable latent image in an area the size of one developed grain). If more than one electron hits a silver halide crystal, only one developed grain will result. Therefore, multiple hits in general will reduce sensitivity. Multiple hits will increase sensitivities, however, if the probability of forming a developable latent image is much smaller for a single interaction between an electron and a silver halide crystal than for a multiple interaction. This may be the situation with higher energy radiation or smaller silver halide crystals than used currently. A detailed analysis of the multiple hit problem on the light microscope level recently has been given by Perry (7). The probability (P) of having more

than one electron hit a silver halide crystal is given by $P = 1 - [(1 + n)/e^n]$ where n is the average number of electrons hitting a silver halide crystal. For low energy radiation, the value of n can be calculated to a good approximation by dividing the number of electrons which hit the emulsion per unit area by the average number of silver halide crystals which form a monolayer in that area. The value of n decreases as the number of silver halide crystals per area of monolayer increases. A monolayer of centrifuged Kodak NTE emulsion contains about 400 silver halide crystals per μ^2 ; a monolayer of Ilford L4 emulsion contains about 40 crystals. Thus there is considerably less probability of multiple hits in the finer grained emulsion. With the sensitivity values given here and the above formula, it can be calculated that the probability of multiple hits is less than 10% if the density of developed grains in a tritium radioautograph is less than 4 developed grains per μ^2 with the Ilford emulsion, and less than 20 (Dektol) or 50 (gold-ascorbic) developed grains per μ^2 with the Kodak NTE. For ^{35}S radioautography the corresponding values are two grains per μ^2 with Ilford L4, and six grains per μ^2 using Kodak emulsion (Dektol developed). If the density of developed grains is higher even within small areas, the multiple hit problem becomes rapidly more important since the value of P increases strongly with increasing values of n .

An overlap of developed grains will result in a lower sensitivity since two developed grains in the same area might be counted as one. In electron microscope radioautography this factor has less influence on sensitivity than multiple hits. The explanation for this is that even the most sensitive emulsion layers have a grain yield considerably smaller than unity, and in high resolution techniques the developed grains occupy an area not much larger than the silver halide crystal. In addition, an overlapping of developed grains becomes obvious to the observer.

In our calibration experiments the doses were such that even for Ilford L4 emulsion the probability of multiple hits had no practical influence on the results.

Exposure Time, Storage Conditions, and Specimen-Emulsion Interaction

The influence of these factors on radioautographic sensitivity has not been studied extensively yet. The experiments leading to the results in

Table I purposely were designed to exclude them as far as possible.

However, in previous studies it was found that Ilford L4 emulsion showed little latent image fading when stored in dry air for 2 months (1, 3, 4). Kodak NTE emulsion must be kept in an inert gas atmosphere in order to minimize this effect.

It also has been reported that (1, 3) the influence of the section on emulsion sensitivity can be eliminated, to a large extent, by the application of an intermediate layer of evaporated carbon between the section and emulsion. These findings are now being reinvestigated quantitatively using the calibration specimen.

Accuracy

An estimate of the accuracy which can be expected for quantitative electron microscope radioautography can be obtained from the reproducibility of the calibration experiments. This was found to be about $\pm 20\%$. The reasons for this variability are not known precisely. Among them probably are errors in judging emulsion thickness and variations in developing conditions. However, one would expect both emulsion thickness and developing conditions to cause considerably smaller variations than those observed. This indicates the influence of some uncontrolled parameters. Frequently poor sensitivities were obtained with the gold latensification—Elon ascorbic acid procedure, even when solutions were freshly made up. Ascorbic acid and, especially, gold chloride seem to have limited self-life, even when stored as solids. Therefore, checking sensitivity before applying this procedure to the radioautographic specimen is important for reproducible results.

When the specimens were stored for long periods, i.e. more than 2 months, after exposure, a greater fluctuation was observed with both emulsions. These fluctuations have not been evaluated quantitatively yet. In addition a rapid increase in background and loss in sensitivity were observed in the Kodak NTE. The use of the Ilford L4, therefore, has an advantage for quantitative work with prolonged exposures, in spite of the expected loss of resolution.

CONCLUSION

The number of external factors which influence sensitivity is endless, and it seems a hopeless task to determine the effect of all possible combina-

tions. Each experiment may introduce unique conditions of its own, i.e., fixation, stain, radioactivity, etc. Furthermore, environmental conditions vary considerably from laboratory to laboratory, and manufacture of the very finely grained emulsions has not settled yet on a desirable level. Even the use of elaborate procedures to ensure constant conditions does not guarantee to overcome all variations, and no blind following of rigorously defined procedures can replace completely criteria for evaluating the results.

In quantitative radioautography, therefore, repeated spot checks of sensitivity are necessary. The stability over long periods also must be tested. The use of sections from radioactive methacrylate blocks, in situations in which tissue-emulsion interactions are not of concern, may be sufficient. For best results, sensitivity should be retested under conditions simulating those of each experiment. One way to do this is to store with the experimental slides during their exposure period emulsion-coated nonradioactive sections that had been irradiated previously with a calibration specimen as described above. An alternative to this is

the use of a calibration specimen with radioactivity comparable to that of most biological specimens. The nonradioactive tissue would then be irradiated by the calibration source throughout the experimental exposure period. In both these cases grain counts over the tissue should give reliable sensitivity values relevant to the conditions of the experiment in question.

The discussion shows that electron microscope radioautography now can be applied for absolute quantitation since all the information needed can be obtained. The section thickness and its variations can be measured interferometrically, the emulsion thickness over the section can be judged by the interference color, and the absolute sensitivity can be measured as described. This method has recently been applied to a quantitative study of intracellular enzyme distribution (11).

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