RESOLUTION IN ELECTRON MICROSCOPE RADIOAUTOGRAPHY

II. Carbon^{14}

M. M. SALPETER and E. E. SALPETER

From the Department of Applied Physics and the Section of Neurobiology and Behavior, and the Laboratory of Nuclear Studies, Cornell University, Ithaca, New York 14850

ABSTRACT

Experimental resolution values, half distances (HD), were determined for electron microscope radioautography with ^{14}C as the source of radioactivity. These were about a factor of 1.5–2 times higher than for tritium. Grain distributions normalized in units of HD were found to fit the "universal" curves previously obtained for tritium.

Most electron microscope (EM) radioautographic studies have employed tritium as the radioactive source. This was aimed at optimizing both resolution and sensitivity; yet it has become clear from theoretical discussions as well as from preliminary experimental results that higher energy isotopes can be used profitably in EM radioautography. In fact, mainly due to the thin tissue sections and emulsion layers of this technique, the relative loss in resolution when going from tritium to higher energy emitters is expected to be considerably less than in light microscope techniques (Bachmann and Salpeter, 1965; Caro and Schnos, 1965). The loss in sensitivity is, however, greater (Bachmann and Salpeter, 1967).

In a recent paper (Salpeter et al., 1969) we described a calibration specimen with the use of tritium as a radioactive source, and presented experimental resolution values for EM radioautography under different geometric and photographic conditions. From the experimental values we extrapolated to expected grain distributions around various radioactive structures, and showed how these could be used for analyzing radioautographs.

In the present study, a similar approach is taken to EM radioautography with ^{14}C as the source of radioactivity. Since the energy distribution from ^{35}S is essentially the same as from ^{14}C (E_{max} of ^{35}S = 167 kv; of ^{14}C = 155 kv), the data presented here are applicable to EM radioautography with ^{35}S. A prediction for even higher energies is also included in the Discussion.

MATERIALS AND METHODS

Calibration Specimen

The calibration specimen was essentially as described for tritium in the papers by Salpeter et al. (1969) and Bachmann et al., (1968). A thin film about 500 A thick of styrene-^{14}C (obtained from New England Nuclear Corp., Boston, Mass., at initial specific activity of 19 μCi/mg) was sandwiched between Epon 812 and methacrylate (specimen 1). When sectioned at right angles it provided a line source of known and uniform label throughout the thickness of the section. The line is visible in the electron microscope without additional staining (Fig. 1).

Since the range of electrons from ^{14}C is considerably greater than that from tritium,[1] it was expected that the tails of the density distributions for the two

[1] From Bethe and Ashkin (1953) one can derive the range of electrons in plastic, which is $\sim$50 μ mean

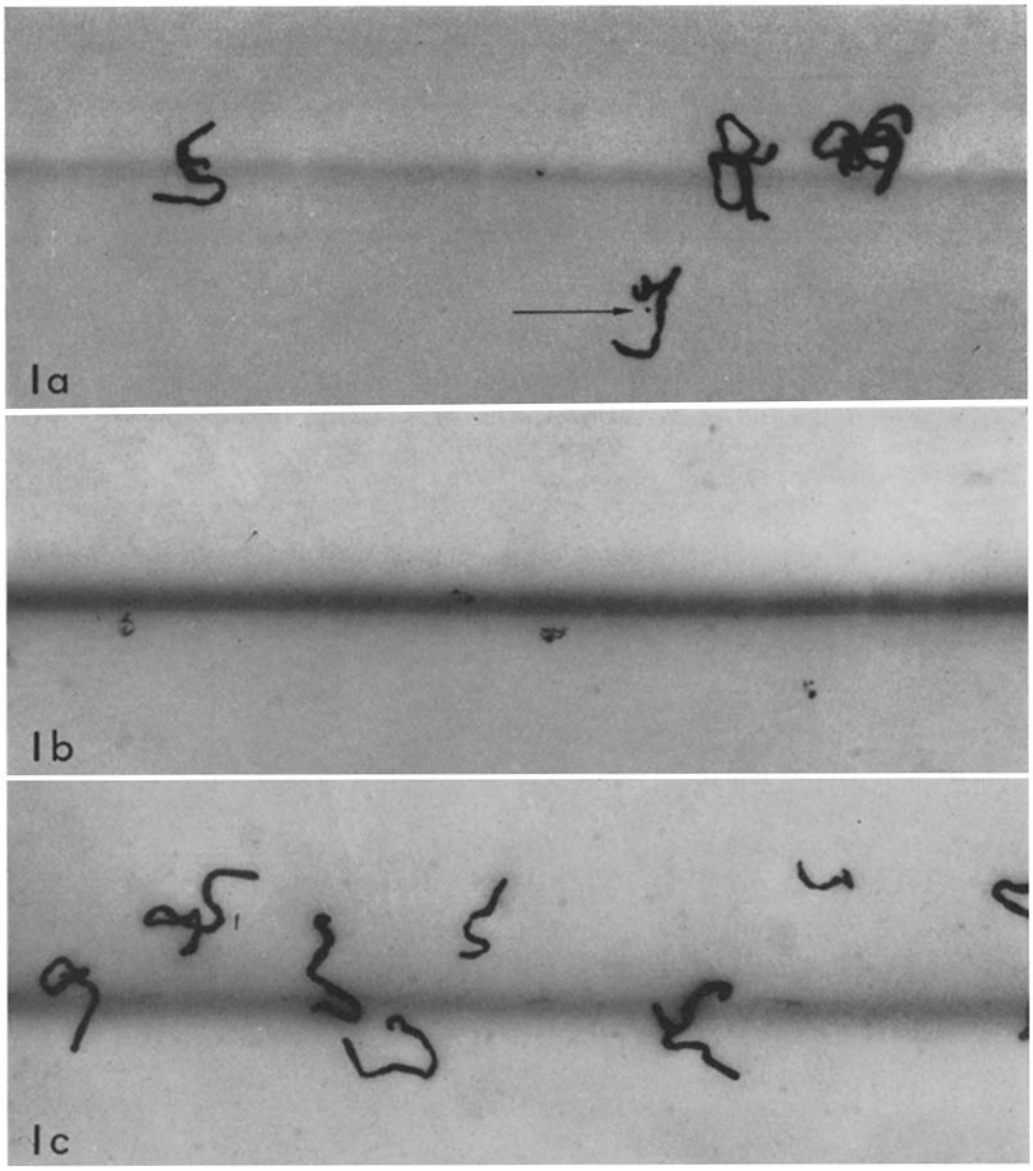

FIGURE 1 Radioactive "hot line" for ^{14}C. Fig. 1 *a*, grey section coated with Ilford L4 monolayer, developed with Microdol-X; Fig. 1 *b*, gold section coated with Kodak NTE double layer, developed with Dektol; Fig. 1 *c*, gold section coated with Ilford L4 double layer, developed in Microdol-X. The location of each grain was determined by its center (e.g. arrow). × 30,000.

isotopes would differ. To improve the statistical accuracy of grain counting at distances > 2 μ from the source, where the grain density is already very low, a second specimen with a 1300 A thick styrene film was used (specimen 2).

and 350 μ maximum for ^{14}C as compared to 0.8 μ and 5 μ, respectively, for ^{3}H.

Experiment

Sections, carefully selected for showing either grey or gold interference colors, were prepared for radioautography by the "flat substrate" method of Salpeter and Bachmann (1964, see also Salpeter, 1966). The grey sections measured on the average 500 A in thickness, and the gold sections were ap-

TABLE I

Specimen		Half distances (HD)	
Emulsion	Section* thickness	Experimental	Extrapolated‡
		A	*A*
Ilford L4 (double thickness; red layer)	gold	2850	
	silver		2650
	grey		2350
Ilford L4 (monolayer; purple layer)	gold	2300	
	silver		2100
	grey	1800	
Kodak NTE (double thickness; purple layer)	gold	2500	
	grey		2000
Kodak NTE (monolayer; pale gold layer)	gold	2000	
	grey		1500

* The extrapolated values represent a linear extrapolation based on the experimental 500 A shift from grey to gold section in the Ilford L4, monolayer specimens, and on our previous findings with tritium.

‡ Gold, silver, and grey sections are approximately 1000 A, 800 A, and 500 A thick, respectively.

proximately twice that thickness. The sections were then coated with carbon and then with a single or double layer of Ilford L4 (Ilford Ltd., Ilford, Essex, England) or of centrifuged Kodak NTE emulsion (Eastman Kodak Co., Rochester, N.Y.). Thicknesses of the emulsion layers were judged by their interference colors (Bachmann and Salpeter, 1965). Their thickness was also recalibrated with the use of a Nomarski incident light interferometer (attached to Reichert light microscope). With Ilford L4 a monolayer has a purple interference color and measures ~1400 A in thickness, and a double layer has a red color and is ~2800 A thick; with Kodak NTE a monolayer has a pale gold color and is ~700 A thick, and a double layer has a purple color and is ~1400 A thick. We define a monolayer as one with a close-packed single layer of silver halide crystals (judged by electron microscopy).

Ilford L4–coated specimens were developed with Microdol-X (Eastman Kodak Co., Rochester, N.Y.), and Kodak NTE specimens with Dektol (Eastman Kodak Co.) (see Fig. 1).

The resolution and relative grain yields of several specimens were determined, and the grain distributions were compared with those obtained for tritium given by Salpeter et al. (1969).

RESULTS

Density Distribution

Radioautograms were analyzed in the same way as those from the ^{3}H-labeled line source (Salpeter et al., 1969), with minor variations. Due to the longer range of ^{14}C, grains were counted over a larger distance from the source. Grains were counted up to 2 μ from the thin line (specimen 1), and between 1 μ and 5 μ from the thicker line (specimen 2). At least 500 grains were collected from each specimen for each of the different geometric conditions reported in Table I. Composite density (grains/area) histograms, combining data from specimens 1 and 2, were drawn, with the overlap region (1–2 μ) insuring that no systematic errors had been made. Background grain density at a distance > 5 μ from the line was determined and subtracted from the experimental values. A sample density distribution comparing ^{14}C with ^{3}H for specimens of similar geometry is given in Fig. 2.

Experimental HD Values

As in the case for tritium, half distance (HD) was again defined as that distance from the center of the line source within which half of the grains were found. This is an integrated value and should not be confused with a density definition of resolution. Furthermore, it must be remembered that HD refers to a line source, the comparable value for a point source (i.e. the radius of a circle around such a point source containing

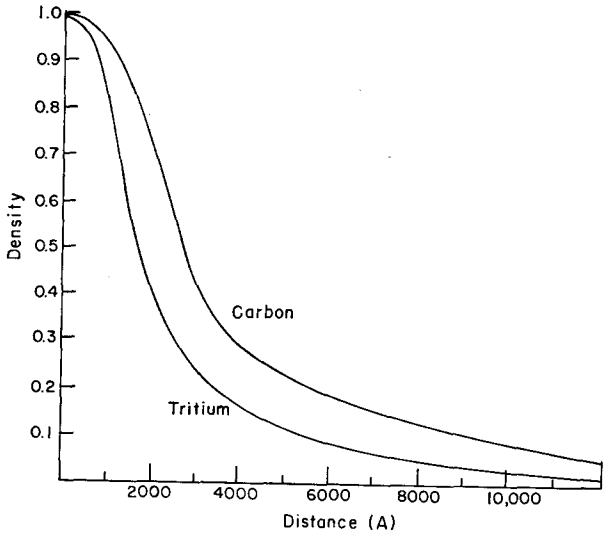

FIGURE 2 Sample experimental density distributions comparing ^{14}C to ^{3}H for comparable section thickness and emulsion-developer combination. Distance from the source is tabulated in Ångstrom units; density is normalized to unity over the line. Gold section, monolayer of Ilford L4, Microdol-X development.

half of the developed grains) being about 1.5 HD (Salpeter et al., 1969).

A provisional experimental half distance value was first obtained from the grains collected within 1 μ of the line. 10 times this value provided the final "cut-off distance" within which grains were tabulated and from which the final experimental HD value was obtained.[2] A small correction for the finite thickness of the line was then made. HD values to the nearest 50 A are reported in Table I.

The accuracy of the HD values for tritium (Salpeter et al., 1969) was estimated to be $\pm$100 A. The ^{14}C values given in Table I are estimated to have an accuracy of $\pm$200 A (percentage-wise similar to that for tritium, or $\sim$10%).

The ^{14}C HD values are larger than those for ^{3}H for comparable radioautographic geometric and photographic conditions, but by less than a factor of two. It is noteworthy that the increase in HD was relatively greater for the Kodak NTE–coated sections than for those coated with Ilford L4. In fact, for specimens of identical geometry, there is no longer any advantage in resolution in using NTE (e.g. compare the gold section coated with a double layer of NTE with that coated with a monolayer of Ilford L4). The absence of scattering by the emulsion of the higher energy electrons emitted from the ^{14}C specimen causes the geometric factors to become paramount. The relatively small photographic advantage thus loses its importance (see Bachmann and Salpeter, 1965, and Bachmann et al., 1968, for a discussion of the relative importance of geometric and photographic factors). Of course, for a monolayer of NTE one still has a better resolution, although a considerably lower sensitivity, (about one-third) compared to a monolayer of L4.

Sensitivity

Sensitivity (or efficiency) in EM radioautography has been defined as the ratio of developed

[2] The "cut-off" distance must be larger than the HD in order to obtain a meaningful value (see Fig. 4). Whur et al. (1969) have recently claimed that our tritium HD values are too large, and that $\sim$98% of all grains would fall within 2250 A from a tritium point source (rather than $\sim$50% as predicted by us; see Fig. 13 *a*, curve p, Salpeter et al., 1969). In evidence they use a histogram (Fig. 8 from Caro, 1962) in which grains are counted only up to 3000 A. It is obvious that had this histogram presented grains only up to 2250 A, then 100% of all grains would have fallen within this distance without making us any wiser regarding the resolution of the specimen.

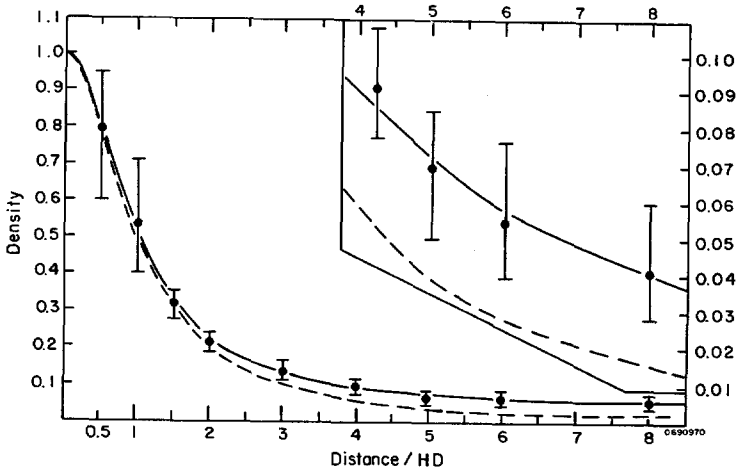

FIGURE 3 Several mean values of the normalized experimental density distributions are plotted against distance/HD. The cross-bars represent the maximum range of experimental deviations from these means. The solid curve represents the best fit for the experimental density distribution and the dashed curve the best fit to the tritium distribution.

grains to radioactive decays in the specimens (see Bachmann and Salpeter, 1967).

Since the energy of radiation from ^{14}C and ^{35}S is essentially the same, the absolute sensitivity values of the latter isotope (i.e. 1/21 for Ilford L4 monolayer, Microdol-X developed) are applicable to ^{14}C. An approximate value obtained from the ^{14}C specimen is 1/25, and for Kodak NTE monolayer is 1/70. The sensitivity appeared to be linear with increasing emulsion thickness. This is not surprising since the range of median electrons from ^{14}C is $\sim 10\ \mu$ in silver halide.

With multilayering, Kodak NTE emulsion has an advantage over the Ilford L4, even though it is not in the realm of resolution. For similar emulsion thickness (i.e. a double layer of NTE *versus* a single layer of Ilford L4) the grain yield is the same, yet the smaller developed grains are less likely to obscure underlying fine structure, and saturation of the emulsion (i.e. one silver halide crystal being hit more than once) presents much less of a problem (see Bachmann and Salpeter, 1967, for discussion). The poor stability of the NTE emulsion with exposures longer than about 2 months, however, makes this emulsion useful only for specimens of high radioactivity.

Normalized Curves

As with tritium, all of the experimental density curves had the same general shape, the extent of spread of each being directly dependent on its HD value. It was, therefore, again convenient to normalize the distance in units of HD, i.e. on the x-axis we plotted distance from the source in Ångstroms divided by HD. When distance was normalized in this manner and density was normalized to be unity at zero distance, all of the density curves appeared almost coincident, giving a universal distribution. In Fig. 3 some average values of the normalized experimental distributions are shown. The best fit for the experimental distribution is given by the solid curve. The dashed curve is the equivalent best fit for tritium, taken from Salpeter et al. (1969). Fortunately, the ^{14}C universal curve normalized in units of HD matches closely that for tritium. This allows the families of curves generated for tritium to be used also for ^{14}C (or ^{35}S) with only a relatively small error (see Discussion).

As discussed in Salpeter et al. (1969), it is useful to look at the integrated (summed) curves which give the relative number (fraction) of total grains up to any given distance from the source. Fig. 4 shows the experimental mean integrated values for ^{14}C plotted against distance in units of HD. The solid curve is a curve of best fit, and the dashed curve the expected distribution for tritium. (By definition the relative number rises to 0.5 at 1 HD and to 1 at 10 HD.) The integrated curve demonstrates the almost linear rise of relative

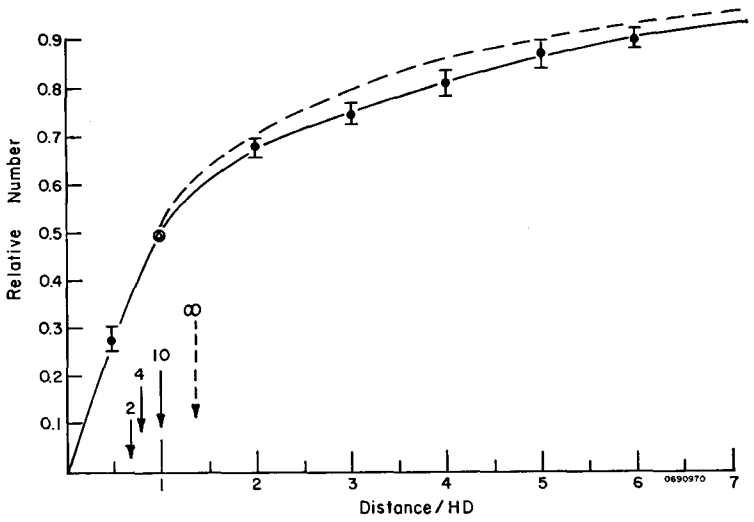

FIGURE 4 This graph plots the same data as in Fig. 3 but in integrated form. Arrows indicate expected HD values for cut-off distances of 2 HD, 4 HD, 10 HD, and infinity, respectively.

number at short distances < 2 HD from the source. This emphasizes the fact that, unless grains are counted at least to where the curve begins to flatten, no meaningful information on percentages of total grains can be obtained.[2]

DISCUSSION

Comparison of ^{14}C and ^{3}H Resolution

In the paper by Salpeter et al., (1969), we discussed problems of resolution in EM radioautography with tritium as the radioactive source. We obtained experimental values for half distance (HD) for a variety of radioautographic conditions. From this experimental information, a family of universal curves was generated which describe expected grain distributions around radioactive sources varying in shape and extent of labeling. When HD values are used as units of distance in analyzing radioautographs, the universal curves can be used to describe the distribution of radioactivity in the tissue. In the present study, experimental values for HD were determined for ^{14}C, and are presented in Table I. (These values are also applicable to ^{35}S because of the equivalence in energy.) The experimental ^{14}C density distribution, normalized in HD units, is given in Fig. 3 and is quite close to the distribution for tritium (dashed curve; also Fig. 4 of Salpeter et al., 1969). In fact, the distributions are almost indistinguishable except that the tail of the distribution is slightly larger for ^{14}C, i.e. for distances beyond about 3 HD the densities are systematically larger for ^{14}C. The universal curves seen in Salpeter et al. (1969), Figs. 9–12, for expected density distributions derived for tritium can thus also be used for ^{14}C without incurring an unduly high error. The slight discrepancy in the tails of the ^{14}C *versus* the tritium curves is of little practical importance in testing for a suspected major radioactive source, where grains densities are rarely needed beyond about 3 or 4 HD. (The ^{14}C and ^{3}H curves are, of course, similar only when all distances are expressed in units of HD, which are almost twice as large for ^{14}C as for tritium. Curves with distances expressed in Ångstroms would naturally be quite different, as illustrated by our Fig. 2.)

The mean experimental values for ^{14}C for relative number (integrated curve) are given in Fig. 4, again with distances expressed in units of HD, and can be compared with the tritium curve (dashed curve; also Fig. 5 of Salpeter et al., (1969). By definition and normalization both curves give values of 0, 0.5, and 1 at distances of 0, 1 HD, and 10 HD, respectively, and are almost indistinguishable at all distances between 0 and about 2 HD. The only noticeable difference is that the ^{14}C curve gives relative numbers lower by about 0.05 for distances between about 3 HD and 5 HD. The derived

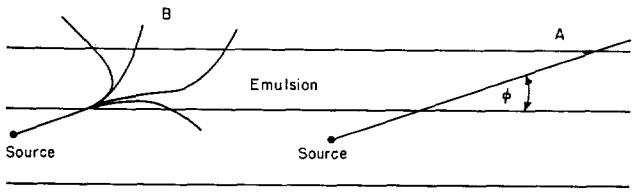

FIGURE 5 Schematic diagrams for possible paths of electrons from a radioactive source in thin biological material. *A* represents no scattering in the emulsion (high energy electrons), and *B* represents possible paths due to scattering (low energy electrons). In both cases the electron leaves the source at a grazing angle (ϕ) to the emulsion surface. For the unscattered (case *A*), the pathlength of the electron in the emulsion is extremely long compared with the thickness of the emulsion. This results in a high probability of forming developed grains at considerable distances from the source. For case *B*, scattering upward both decreases the path length and tends to return the electron towards the source. Scattering downward could lead to an increased pathlength but (due to the initial small angle ϕ) often leads to the loss of the electron out of the emulsion. The net effect of scattering is thus to decrease the relative number of developed grains with distance from the source.

universal curves (Salpeter et al., 1969, Figs. 13–15) for relative number can thus also be used for analyzing ^{14}C radioautograms without incurring excessive additional errors (i.e. errors no bigger than those due to uncertainties of about $\pm 10\%$ in the numerical value of HD).

We estimate that about 10–20% of all grains fall beyond our cut-off distance of 10 HD (compared with about 5–10% for tritium). However, these grains are of little importance for most analyses, because they represent grain densities which are both very small numerically and change very slowly with distance. In practice, such grains merely make a small contribution to an almost uniform "background" density. Such background density, of course, makes it difficult to determine the extent of label in areas with low radioactivity, even within relatively large distances from intensely labeled structures.

Predictions for Higher Energy Isotopes

When beta-emitting isotopes which have even higher energies than ^{14}C and ^{35}S (such as ^{45}Ca) are to be used, separate measurements will have to be made to determine the resultant further loss of sensitivity and resolution. We can, however, estimate (at least qualitatively) on theoretical grounds the effect on resolution of very high energy electrons.

Even with ^{14}C most of the electrons have sufficiently high energy to traverse section and photographic emulsion essentially in a straight-line path (i.e., see Fig. 5, *A*) without scattering. Only about 10% of the electrons with the lowest energy behave roughly like tritium electrons, and are scattered significantly in the emulsion (i.e. deflected from a straight line, see Fig. 5, *B*). In addition, scattering is also a factor for the few electrons which are emitted from the source in a direction almost parallel to the section surface and thus have a long path length in the emulsion. As can be seen from Fig. 5, *B*, scattering tends to decrease the spread of radiation from the source and thus to decrease HD. This is the reason why HD increases with increasing electron energy.

The main effect, therefore, of going to isotopes of even higher energy than ^{14}C is simply to decrease the importance of scattering even further. Even those electrons travelling almost parallel to the surface will not be scattered and thus they could produce developed grains very far from the source. The tail of the density distribution will therefore fall off even more slowly than for ^{14}C. Once an energy level is reached for which there is no longer any scattering in the emulsion, further increases in energy will not alter the density distribution. This would be equivalent to the nonscattering $\cos^2\theta$ case for a point source discussed by Bachmann and Salpeter (1965). We expect this limit to be reached with emulsion layers used in electron microscope radioautography at an energy two to three times that of ^{14}C.

As already discussed, the "tail" of the density distribution raises the general grain background but does not seriously affect grain density near the source. Therefore, to obtain a useful HD value for high energy beta emitters one should disregard the terminal flat part of the long tail by, for in-

stance, subtracting it from the density distribution. One will then obtain values for HD only slightly higher (~10–20%) than those for ^{14}C in our present Table I. This will enable one again to use the "universal curves" from Salpeter et al. (1969). Unfortunately, with high energy sources there will always be a residual uncertainty as to whether an extended area of low grain density represents real label or merely radiation spread from surrounding sources. For quantitative work the grain density over such areas will have to be regarded as tissue background and be subtracted out.

Estimated Effect of Backscatter

In the "flat substrate" technique used in the present investigation as well as in Salpeter et al. (1969), the section rests on a glass slide with approximately 350 A of collodion in between. It is of some interest to estimate theoretically what effect the presence of the glass has on electron backscatter.

Electrons are incident onto the glass from the section via the collodion film at various angles and with various energies. Some of these electrons are slowed down sufficiently in the glass to come to rest completely in the glass; others suffer some energy loss but are multiply scattered and finally escape from the glass back into the collodion film and then to the emulsion. The fraction of electrons incident on a slab of material which are backscattered has been measured as a function of electron energy, incident angle, and composition of the material (Sternglass, 1954; Kanter, 1957). This fraction depends very little on electron energy for energies of about 2 kev and larger, and should therefore be almost the same for tritium electrons, ^{14}C electrons, and those from higher energy sources. For glass the fraction of all the electrons incident at all angles which are backscattered should be about 30%. The backscatter measurements also indicate that the backscattered electrons lose about 40% of their energy in the process.

Although the fraction of electrons backscattered from a glass slab is almost independent of electron energy, the practical effect of backscatter is nevertheless different for tritium and higher energy electrons. This is in part due to the fact that backscattered electrons emerge from the glass at a point whose horizontal distance from the point of entry is approximately half the range of the electron in glass. This distance is thus larger for higher energy electrons. For ^{14}C, electrons penetrate the collodion film and section easily and appear in the emulsion between about 5 μ and 50 μ away from the source. Because of these large distances (all larger than 10 HD) these backscattered electrons have a negligible effect on the density curve. On the other hand, they represent an additional 30% of total developed grains appearing essentially as an increased smooth background over a large area, thus of low density. (For higher energy emitters with distances from the source even larger, the situation is qualitatively similar.)

The situation is different for tritium electrons because of their lower energy and much shorter range. For electrons to backscatter from the glass and then reach the emulsion, they must first traverse the section and collodion, reach the glass, backscatter which lowers their energy by about 40%), and then traverse the whole system again. The probability is high that they will be absorbed in the process and that only about one-third as many as for ^{14}C will survive and reach the emulsion. Thus, only about 10% of all developed grains for tritium are estimated to be due to electrons backscattered from the glass. These grains will be spread mainly at distances between 0.5 μ and 2 μ from the source and may account for the slight discrepancy between the tail of the theoretical density distribution and the experimental curve for tritium discussed in Salpeter et al. (1969).

We wish to thank Dr. Luis Bachmann for help in the early stages of this study, Dr. Isabelle Faeder for helpful discussions, and Mrs. Frances McHenry for technical assistance.

This work was supported in part by the United States Public Health Service Research Grant GM-10422 from the Division of General Medical Sciences and by Career Development Award NB-K3-3738 from the Division of Neurological Diseases and Stroke and by United States National Science Foundation Grant GP20006.

Received for publication 30 October 1970, and in revised form 6 January 1971.

REFERENCES

BACHMANN, L., and M. M. SALPETER. 1965. Autoradiography with the electron microscope: a quantitative evaluation. *Lab. Invest.* **14**:1041.

BACHMANN, L., and M. M. SALPETER. 1967. Absolute sensitivity of electron microscope radioautography. *J. Cell Biol.* **33**:299.

BACHMANN, L., M. M. SALPETER, and E. E. SALPETER. 1968. Das Auflosungsvermogen elektronen-

mikroskopischen Autoradiographien. *Histochemie.* **15**:234.

BETHE, H. A., and J. ASHKIN. 1953. Passage of radiation through matter. *In* Experimental Nuclear Physics. E. Segre, editor. John Wiley and Sons, Inc., New York. **1**:166.

CARO, L. G. 1962. High resolution autoradiography. II. The problem of resolution. *J. Cell Biol.* **15**:189.

CARO, L. G., and M. SCHNOS. 1965. Tritium and phosphorus-32 in high resolution autoradiography. *Science (Washington).* **149**:60.

KANTER, H. 1957. Zur Ruckstreuung von Electronen im Energiebereich von 10 bis 100 keV. *Ann. Phys. (Leipzig).* **20**:144.

SALPETER, M. M. 1966. General area of autoradiography at the electron microscope level. *Methods Cell Physiol.* **2**:229.

SALPETER, M. M., and L. BACHMANN. 1964. Autoradiography with the electron microscope. A procedure for improving resolution sensitivity and contrast. *J. Cell Biol.* **22**:469.

SALPETER, M. M., L. BACHMANN, and E. E. SALPETER. 1969. Resolution in electron microscope radioautography. *J. Cell Biol.* **41**:1.

STERNGLASS, E. J. 1954. Backscattering of kilovolt electrons from solids. *Phys. Rev.* **95**:345.

WHUR, P., A. HERSCOVICS, and C. P. LEBLOND. 1969. Radioautographic visualization of the incorporation of galactose-^{3}H and mannose-^{3}H by rat thyroid in vitro in relation to the stages of thyroglobulin synthesis. *J. Cell Biol.* **43**:289.