Chapter 13

Autoradiography with the Electron Microscope: Properties of Photographic Emulsions

D. F. HÜLSER[1] AND M. F. RAJEWSKY[1]

Max-Planck-Institut für Biophysik, Frankfurt/Main, Germany

I. Introduction

Autoradiography, namely the registration and localization of radiation from radionuclides by photographic emulsions, has been used in biological experimentation since the early studies of Lacassagne and Lattès (1924) who demonstrated the presence of Po^{210} in rabbit organs by this method. (For early literature see L. G. Caro, Vol. I, this treatise.)

[1] *Present address:* Abteilung für Physikalische Biologie, Max-Planck-Institut für Virusforschung, Tübingen, Germany.

In recent years, a great number of compounds of biological interest labeled with low energy β-emitting radionuclides have become available and, consequently, the number of biological investigations using autoradiographic methods has increased rapidly. This is also reflected in several contributions in Vols. I and II of this treatise (Caro, 1964; Miller *et al.*, 1964; Perry, 1964; Prescott, 1964; Salpeter, 1966; Stevens, 1966).

In combination with light or electron microscopy, autoradiographic techniques are used for the localization of label within tissues and cells as well as for comparative measurements of the incorporated radioactivity. However, autoradiographic results are often unsatisfactory with regard to quantitation and reproducibility. This is partly due to the nature of the methods and can hardly be completely eliminated. For example, it is impossible to reestablish the original distribution of AgBr grains after development.

Besides these shortcomings, errors in the interpretation of autoradiographic results are often caused by insufficient consideration of the photographic elementary processes and the characteristics of the emulsions used.

In this chapter, some current techniques of autoradiography with the electron microscope will be discussed, as well as some properties of the three photographic emulsions most commonly used for this purpose, namely, Gevaert NUC 307, Ilford L4, and Kodak NTE.

II. Techniques for Autoradiography with the Electron Microscope

The preparation of labeled biological specimens for autoradiography does not differ from the usual techniques employed in electron microscopy. This means that the labeled compounds bound to the biological material must not be soluble in the solutions used for processing the specimens. The risk of chemical artifacts during long periods of exposure and during processing can be reduced by using platinum grids. The specimens, usually ultrathin sections of embedded biological material, are applied to Formvar- and carbon-coated grids. To avoid chemical interactions between section and photographic emulsion, the preparations are then coated with another carbon layer (Salpeter and Bachmann, 1964).

A convenient technique for the application of photographic emulsion to the specimen has been reported by Caro and van Tubergen (1962), and is employed in our laboratory in a slightly modified form. The emul-

sion is diluted to a suitable degree (about 1:4) with distilled water, heated to 40°C, and stirred continuously with a glass magnetic stirrer for 30 minutes. By the addition of detergent (for example Hostapon, Farbwerke Hoechst AG) (Haase and Jung, 1964), the surface tension is reduced to allow a very thin membrane of emulsion to be formed within a copper wire loop when dipped into the liquid emulsion and withdrawn slowly. The loop is fixed about 20 cm above the magnetic stirrer plate at an angle of 45° and dried for about 2 minutes in this position. Immediately after the emulsion membrane has gelled it is carefully brought in contact with the specimen by a quick breath. The zone of optimum emulsion thickness is one which contains a monolayer of AgBr grains, and its position within the loop can be found by a series of test grids from different areas of the gelled emulsion membrane. The loop should be even and either circular or oval, with a diameter of about 3 to 5 cm. An "expandable" loop has been described by Karasaki (1965). For exposure the specimens are then stored at 4°C in light tight Bakelite containers to which a small amount of Drierite is added. After an exposure time which depends on the degree of labeling (14–60 days), the preparations are developed. The problem of development will be discussed in Section III,B.

III. Theoretical Considerations

A. Resolution

In autoradiography, an electron source does not lead to a reproducible image in the optical sense, but is represented by developed AgBr grains (silver filaments) whose occurrence is only reproducible within a certain area around the source. The location of the source must, therefore, be deduced from the position of the silver filaments. Since a source emitting only one single electron cannot in practice be detected in biological material, so-called "point sources" always emit a number of electrons and are, therefore, usually represented by more than one silver filament. Autoradiographic resolution, when defined as the separation of two adjacent sources, depends on the number of silver filaments per unit area produced by the respective source. Under optimum conditions autoradiographic resolution is of the order of 0.1 μ (Caro, 1962). The following factors determine the yield of silver filaments and autoradiographic resolution: (a) grain size, grain size distribution, sensitivity, and the density of the monolayer; (b) energy and range of the radionuclides;

(c) thickness and surface quality of the biological sections and contact with the emulsions; and (d) conditions for development.

The conclusion could be drawn that resolution increases with decreasing grain size. However, as will be shown in Section IV,B, for smaller grains more than one electron is necessary to induce a latent image (Hülser and Rajewsky, 1966). It is, therefore, necessary to obtain an estimate of the probability P_t for a single AgBr grain to be hit by one electron emitted from a labeled molecule.

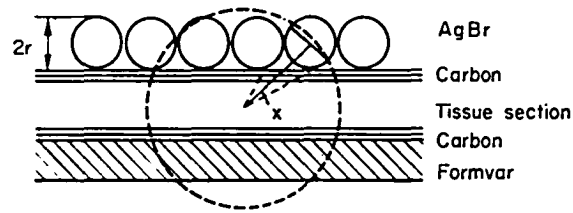

FIG. 1. The probability of latent image formation as a problem of resolution. r, Radius of AgBr grain; x, distance source–center of AgBr grain; circle area, $F_1 = \pi r^2$; sphere surface, $F_2 = 4\pi x^2$; probability for one hit, $P_t = F_1/F_2 = r^2/4x^2$; probability for latent image formation, $P_s = (r^2/4x^2)^i$; i, number of electrons necessary for the formation of one latent image.

In Fig. 1, a point source of electrons is shown, covered by a monolayer of AgBr grains. In this case, the probability for one hit is approximately given by the ratio F_1/F_2, where F_1 is the area of a circle with the radius of the AgBr grain and F_2 is the surface area of a sphere with the radius x (equal to distance from the electron source to the center of AgBr grain).

$$P_t = \frac{F_1}{F_2} = \frac{1}{4} \cdot \frac{r^2}{x^2} \tag{1}$$

However, for emulsions which require several electrons for the formation of a latent image, the probability P_s for the formation of a latent image must be taken into account. P_s is a function of the number of electrons i necessary for the production of one latent image, and of the probability for one AgBr grain to be hit by one electron.

$$P_s = \left(\frac{1}{4} \cdot \frac{r^2}{x^2}\right)^i \tag{2}$$

The validity of this equation is limited to grains in the immediate neighborhood of the source, since energy absorption by these grains can prevent latent image formation in the more distant grains (see also Caro, 1962). For good estimation of P_s, the size distribution of AgBr grains should have a small half-width value.

For thin sections and small but sensitive AgBr grains, resolution is high and increases with decreasing range of the incorporated radionuclides. In Fig. 2, the range of electrons in AgBr is demonstrated for low β-energies. Tritium (maximum energy 18.6 keV), the radionuclide with the lowest energy and range of all β-emitters, is most frequently used in electron microscopic autoradiography, followed by C^{14} (158 keV), S^{35} (167 keV), and P^{32} (1.71 MeV).

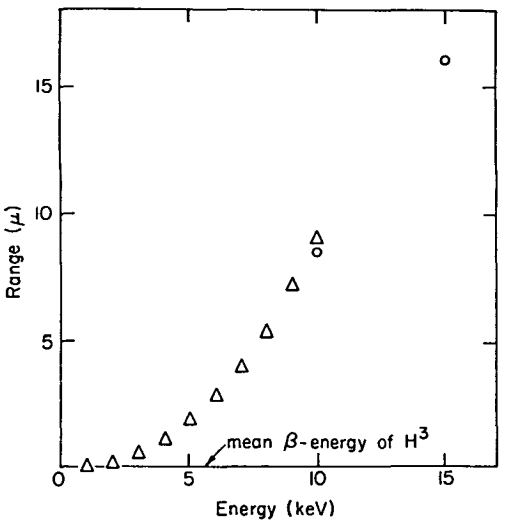

FIG. 2. Range of electrons in AgBr. Δ, Calculated after Feldmann (1960); ○, measured by Berger and Seltzer (1964) and Nelms (1956).

For H^3-labeled preparations, Maurer and Primbsch (1964) have shown that the yield of developed grains increases linearly with the section thickness up to a value of 0.5 to 1 μ for karyoplasm and cytoplasm, whereas for the nucleolus the linearity is only given up to about 0.1 μ. Therefore, the section thickness for microautoradiography has to be a compromise between the desire for a maximum yield of developed grains and optimum quality of the electron microscopic picture, which is satisfactory only for section thicknesses of up to 0.1 μ.

B. Development

The electron energy, absorbed by a grain, induces the formation of latent images at certain lattice sites within the AgBr crystal. Within the grain, the locus of the latent image is not necessarily identical with the site of the electron hit. The size of the latent images is of the order of the diameters of several Ag atomic diameters. Even for demonstration

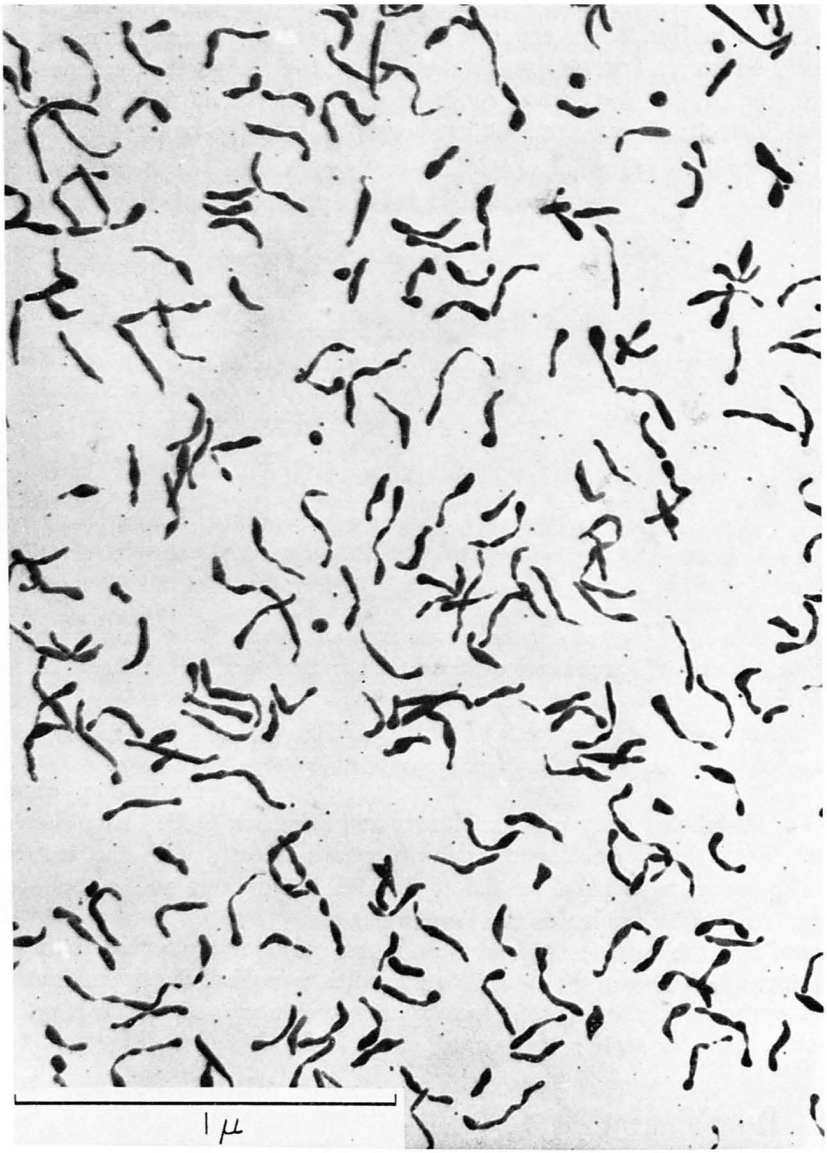

FIG. 3. Ilford L4 emulsion. Irradiation with 5.7 keV electrons in the AEG "SDL" electron microscope. Developed 1 minute in Microdol X. Magnification × 53,000.

in the electron microscope, latent images have to be enlarged (i.e., developed) chemically.

In "chemical" development (see Joos and Schopper, 1958; von Angerer and Joos, 1959), the latent image is rapidly enlarged by addition of Ag which is the result of the reducing effect on AgBr by the developer. Characteristically shaped silver filaments grow out from the AgBr grain. The unexposed AgBr is subsequently removed by fixation.

In "physical" development (see Joos and Schopper, 1958; von Angerer and Joos, 1959), the unexposed AgBr is first removed by fixation, leaving only the latent images intact. These are then enlarged by a developer containing Ag^+ ions. The size of the silver spots obtained by attachment of Ag^+ ions to the latent image is, therefore, a function of the time of development. However, apparently because of the likelihood of artifacts, physical development has not been used so far in autoradiography with the electron microscope.

A third possibility for the demonstration of the latent image is the so-called "fine-grain" development (see Joos and Schopper, 1958; von Angerer and Joos, 1959), which is often misleadingly referred to as physical development. This type of development can be considered as a combination of physical and chemical development. A developer is chosen which also dissolves the AgBr and thereby produces a solution containing Ag^+ ions. In this case the latent image is not only chemically developed by reduced Ag from the same grain, but also by physical attachment of Ag^+ ions from the solution. Under suitable conditions one obtains small and characteristically "tadpole-shaped" silver filaments, in which the finer end represents the locus of the original image. Figure 3 shows such "tadpole-shaped" filaments after development in Microdol X. The developing time depends on the type of developer, but should never be less than 2 minutes at room temperature for a maximum number of latent images to be developed. After stopping the development in acetic acid for 10 seconds, the specimens are fixed for 5 minutes in Kodak AM 33 rapid fixer made up 1:7 in distilled water. The yield of developable latent images can be increased by a procedure called gold latensification (see Salpeter, 1966), where gold ions are deposited on prestages of latent images, which are normally undevelopable.

IV. Determination of the Sensitivity of Photographic Emulsions

The sensitivity of photographic emulsions for autoradiography with the electron microscope can be defined and tested in different ways

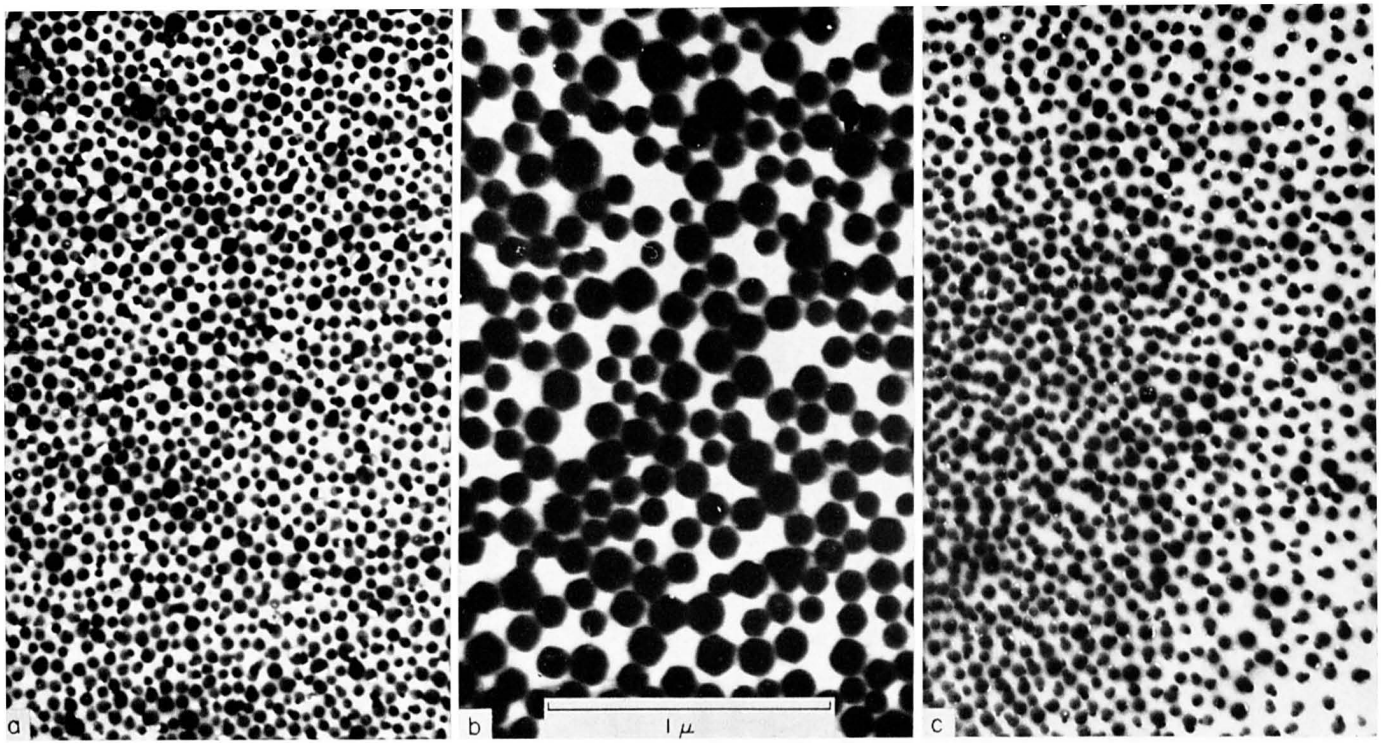

FIG. 4. Part I. See legend on opposite page.

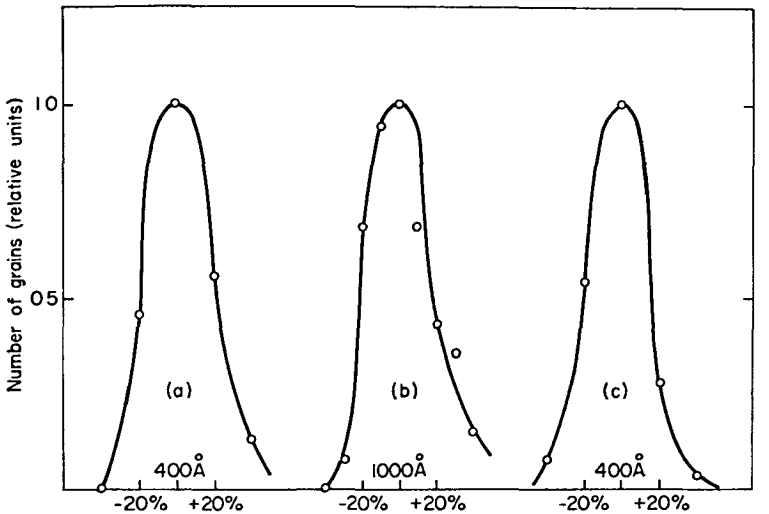

FIG. 4. Silver bromide grains of the emulsions Gevaert NUC 307 (a), Ilford L4 (b) and Kodak NTE (c), and their respective normalized grain size distribution curves. Magnification × 38,000.

(Caro, 1962; Granboulan, 1963; Caro and Schnös, 1965; Hülser and Rajewsky, 1966; Bachmann and Salpeter, 1967). Basically, it is necessary to obtain information on the electron sensitivity of single grains. This problem has been investigated for the emulsions Gevaert NUC 307, Ilford L4, and Kodak NTE. The grain size distribution curves are shown in Fig. 4. As can be seen, all three emulsions have half-width values of about 20%. Apparently, mean grain size and sensitivity of the emulsions may vary somewhat from one batch to the other; for example, the value found for the mean grain size of the Gevaert emulsion was 40% lower than that stated by the manufacturer [see also comments by Bachmann and Salpeter (1967) on the variability of different batches of Kodak NTE emulsion].

A. Methods

Monolayers of these emulsions have been prepared on Formvar- and carbon-coated platinum grids and irradiated in an AEG "SDL" electron microscope under reproducible experimental conditions. Irradiations were carried out with a homogeneous electron flux of about 2×10^{-9} A/cm^2, measured with the use of a Faraday cage. The energy of the electron beam was maintained at 5.7 keV, corresponding to the mean β-energy of tritium. A mechanical cover plate was used to perform step-

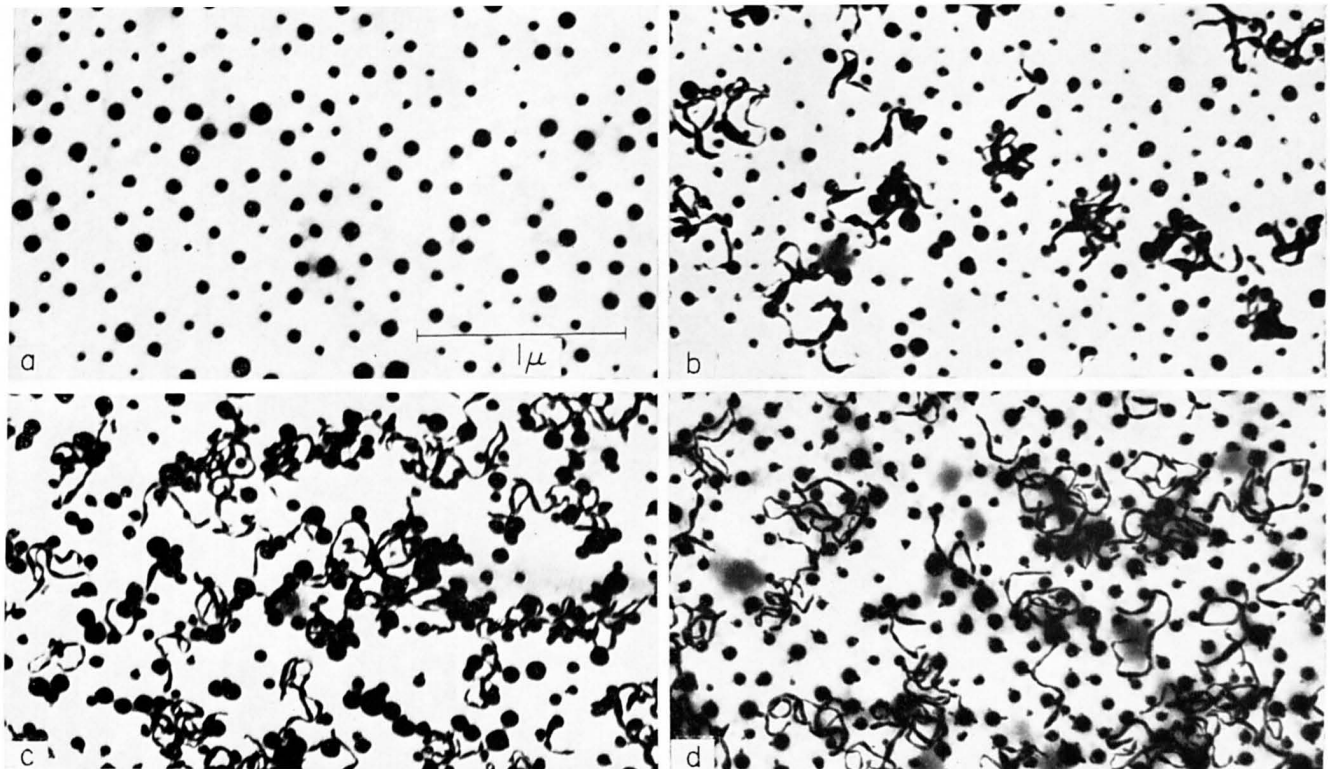

FIG. 5. Ilford L4 emulsion irradiated with 0 (a), 1 (b), 2 (c), and 20 (d) electrons per AgBr grain. Developed in Microdol X for 1 minute, stopped in acetic acid, unfixed. Similar plates were used to determine the proportion $G_N(n)$ of developed AgBr grains. Magnification $\times$ 28,000.

wise exposure of the specimens. For the sensitivity determinations, the dose to which the specimens were exposed was expressed as electrons (e) per AgBr grain (g) (e/g = N). The irradiated monolayers were developed in Microdol X (diluted 1:2 with water) at 22°C for 1 minute, stopped in 1% acetic acid for 10 seconds, rinsed in water, and left unfixed. Undeveloped and developed grains could then be distinguished and the proportion G_N (n) of developed AgBr grains determined (see Fig. 5a–d).

B. Results

The evaluation of the irradiated specimens shows that even in the case of high doses ($N = 20$ e/g) a certain number of AgBr grains was not developable under the experimental conditions used (NUC 307: ≈15%; L4: ≈25%; NTE: ≈10%). Because of the short developing time not all latent images grow out completely, as it takes longer to develop the internal latent images than those on the surface of the grains. On the other hand, the time of development had to be kept short in order to obtain small silver filaments which allow a clear distinction between the single grains. The points, however, where silver filaments are just starting to grow out from the grains are clearly distinguishable. The values obtained after counting the number of developed and undeveloped grains, were normalized with respect to the percentage of nondevelopable grains in the respective emulsions.

If we assume that electrons hit AgBr grains according to a Poisson distribution

$$G_N(n) = 1 - e^{-N} \sum_{n=0}^{k-1} \frac{N^n}{n!} \tag{3}$$

we can calculate the number of grains which are hit by k or more electrons when the mean exposure is N electrons per grain. In Fig. 6 the percentage of developed AgBr grains is given on the ordinate; the number of electrons necessary for the formation of a latent image can be read from the abscissa, using the curve for the respective value of N.

According to our experiments, for the formation of one silver filament, one AgBr grain of the Ilford L4 emulsion must be hit on the average by 1 to 1.4 electrons. The corresponding figures for the Gevaert NUC 307 and Kodak NTE emulsions are 2–3 electrons and 4–5 electrons, respectively.

Integration of the curve in Fig. 2 gives the loss of energy for electrons in AgBr (see also Pelc, 1963). From the resulting value for 5.7 keV elec-

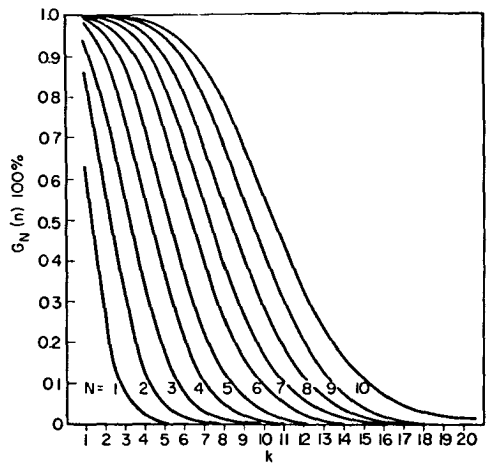

FIG. 6. Poisson distribution curves for the determination of the number of electrons necessary for the formation of one latent image. *N*, Number of electrons to which one AgBr grain was exposed; *k*, Number of electrons hitting one AgBr grain.

trons (mean β-energy of H^3) it can be estimated that the Ilford L4 and Gevaert NUC 307 emulsions absorb about 800 eV for the formation of one latent image as compared with about 1300 eV in the case of the Kodak NTE emulsion. However, it must be assumed that within the grain only part of this energy results in latent image formation (see Malinovski *et al.*, 1964).

V. Discussion

Autoradiographic resolution will, in principle, increase with the number of electrons necessary for latent image formation, thus favoring the emulsions Gevaert NUC 307 and Kodak NTE. This is, however, not a true advantage since, on the other hand, resolution also depends on the yield of developed AgBr grains, which becomes smaller the more electrons are required per AgBr grain. With emulsions requiring several electrons per AgBr grain, little labeling will be obtained. Although the Ilford L4 emulsion has about twice the mean grain size of the Gevaert NUC 307 emulsion, considerations with regard to the probability of latent image formation for one- and multiple-hit processes show that autoradiographic resolution for these emulsions is of the order of 0.1 μ in either case. The Ilford L4 emulsion is, therefore, preferred in our laboratory since with a shorter exposure time results are of comparable

quality. Furthermore, this emulsion seems to be rather constant with respect to grain size and sensitivity. The Gevaert NUC 307 emulsion is apparently subject to variations in grain size (see Section IV). Although on the basis of our findings the sensitivity of the Kodak NTE emulsion is the lowest of the three emulsions in question, results published by Bachmann and Salpeter (1967) suggest that the sensitivity of this emulsion may vary considerably.

According to our present knowledge, an overall absorption of several hundred electron volts per AgBr grain seems necessary for latent image formation by exposure to electrons when normal developing procedures are used (see Malinovski *et al.*, 1964). As mentioned in Section IV,B, not the whole energy absorbed by the AgBr grain can be attributed to latent image formation.

It is generally accepted (see von Angerer and Joos, 1959; Malinovski *et al.*, 1964) that an energy of 30 to 70 eV per AgBr grain is necessary for the formation of one latent image by photons. In the case of electrons, however, only the overall energy absorption has so far been measured. Since the specific energy absorption for photons is not comparable with the overall value for electrons, it is not possible to infer a minimum size of AgBr grains for electron exposure from the value of 30 to 70 eV for photons.

On the other hand, our results show that different numbers of electrons are necessary for latent image formation in two emulsions with almost identical grain size. This indicates that grain size is not the only critical factor for the sensitivity of photographic emulsions. The possibility should therefore be considered that in the future the presently optimum grain size for tritium autoradiography (1000 Å; Ilford L4) could be decreased by the use of suitable sensitization methods. Furthermore, techniques may be developed (as for example gold latensification) to demonstrate prestages of the latent image which remain undetected with the developers presently used.

Acknowledgment

We should like to thank Springer-Verlag, Berlin for permission to reprint Figures 1, 3, upper part of 4, 5, and 6 from *Biophysik* 3, 123–130 (1966).

References

Bachmann, L., and Salpeter, M. M. (1967). *J. Cell Biol.* 33, 299.
Berger, M. I., and Seltzer, S. M. (1964). *Report NASA SP-3012.*
Caro, L. G. (1962). *J. Cell Biol.* 15, 189.
Caro, L. G. (1964). *In* "Methods in Cell Physiology" (D. M. Prescott, ed.), Vol I, pp. 327–363. Academic Press, New York.
Caro, L. G., and Schnös, M. (1965). *Science* 149, 60.

Caro, L. G., and van Tubergen, R. P. (1962). *J. Cell Biol.* **15**, 173.
Feldman, C. (1960). *Phys. Rev.* **117**, 455.
Granboulan, P. (1963). *J. Roy. Microscop. Soc.* **81**, 165.
Haase, G., and Jung, G. (1964). *Naturwissenschaften* **17**, 404.
Hülser, D. F., and Rajewsky, M. F. (1966). *Biophysik* **3**, 123.
Joos, G., and Schopper, E. (1958). "Grundriß der Photographie und ihre Anwendungen besonders in der Atomphysik." Akad. Verlagsges., Frankfurt/Main.
Karasaki, S. (1965). *J. Cell Biol.* **26**, 937.
Lacassagne, A., and Lattès, J. S. (1924). *Compt. Rend. Acad. Sci.* **178**, 488.
Malinovski, I., Todorova, M., and Djambazova, M. (1964). *In* "Photographie Corpusculaire III" (P. Demers, ed.), pp. 75–79. Les Presses Universitaires de Montréal, Montréal.
Maurer, W., and Primbsch, E. (1964). *Exptl. Cell Res.* **33**, 8.
Miller, O. L., Jr., Stone, G. E., and Prescott, D. M. (1964). *In* "Methods in Cell Physiology" (D. M. Prescott, ed.), Vol. I, pp. 371–379. Academic Press, New York.
Nelms, A. T. (1956). NBS Circular 577 and S.
Pelc, S. R. (1963). *J. Roy. Microscop. Soc.* **81**, 131.
Perry, R. P. (1964). *In* "Methods in Cell Physiology" (D. M. Prescott, ed.), Vol. I, pp. 305–326. Academic Press, New York.
Prescott, D. M. (1964). *In* "Methods in Cell Physiology" (D. M. Prescott, ed.), Vol. I, pp. 365–370. Academic Press, New York.
Salpeter, M. M. (1966). *In* "Methods in Cell Physiology" (D. M. Prescott, ed.), Vol. II, pp. 229–253. Academic Press, New York.
Salpeter, M. M., and Bachmann, L. (1964). *J. Cell Biol.* **22**, 469.
Stevens, A. R. (1966). *In* "Methods in Cell Physiology" (D. M. Prescott, ed.), Vol. II, pp. 255–310. Academic Press, New York.
von Angerer, E., and Joos, G. (1959). "Wissenschaftliche Photographie." Akad. Verlagsges. Geest u. Portig KG, Leipzig.