HIGH-RESOLUTION AUTORADIOGAPHY

II. The Problem of Resolution

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ABSTRACT

The resolution obtainable in electron microscopic autoradiographs, using a photographic emulsion consisting of a monolayer of silver bromide crystals, was investigated theoretically and experimentally. The expected distribution of exposed crystals around a point source was calculated from the geometry of the preparation and from the range distribution of the beta particles emitted by tritium. From such a distribution an autoradiographic resolution of the order of 1000 A can be predicted. From the point source distribution, the expected distribution of grains around bacteriophages labeled with tritium was calculated. This distribution was also measured experimentally in electron microscopic autoradiographs of bacteriophages T-2 labeled with thymidine-H^3. The two distributions agreed closely. It was also verified, using the nuclear region in thin cross-sections of *Bacillus subtilis* labeled with thymidine-H^3, that resolutions of the same order were obtained for extended sources. It was concluded that an autoradiographic resolution of 1000 A could be achieved with a presently available commercial emulsion, although emulsions with finer grains might be desirable in some circumstances.

INTRODUCTION

We have, in the preceding article, described general methods for high-resolution autoradiography, with some emphasis on electron microscopic methods. We now propose to examine, theoretically and experimentally, the resolution of which such methods are capable.

In one of the earliest attempts to visualize tracks from radioactive decays at the electron microscope level, Comer and Skipper (1) noticed that the probability for a long track from a single radioactive particle was low, but these authors were not concerned with problems of resolution and did not therefore examine the question further. The first application of electron microscopic autoradiography to biological specimens by Liquier-Milward (2) did not demonstrate a striking increase in autoradiographic resolution. In another early attempt (3) O'Brien and George mentioned that this technique led to improved resolution and showed some evidence for this. Using bacteria labeled with tritiated thymidine and a fine grain emulsion (Kodak V-1055), van Tubergen (4) was the first to demonstrate clearly a resolution superior to that obtained by conventional techniques. By using thin sections of tissue labeled with tritium and thin layers of fine grain emulsions (Ilford K-5 and L-4), we have obtained autoradiographic resolutions at the subcellular level with little loss in image quality (5, 6). Comparable results have been obtained by several authors (7–10).

In 1950 Doniach and Pelc (11) examined theoretically the problem of resolution in autoradiography and calculated the expected dis-

tribution of grains around a point source for various situations. These calculations were confirmed experimentally by Stevens (12). Both articles were mainly concerned with the influence of the spacing between emulsion and specimen. Doniach and Pelc's calculations did show, however, that if the emulsion and the specimen were each 2 μ thick a better resolution was obtained than in the case of a 5 μ specimen and a 15 μ emulsion. If the specimen was 2 μ thick and the emulsion thickness increased from 2 to 5 μ the resolution was practically unaffected. Similarly Nadler (13) concluded that emulsion thickness was not an important factor in achieving optimum resolution. It should be noted that both papers considered the case of isotopes emitting beta particles with ranges considerably longer than those of tritium. Gomberg and Schlesinger (14) used slightly different premises and a different criterion for resolution and reached, without giving the detail of the calculations, the conclusion that optimum resolution will be obtained if the specimen and the emulsion are thin and their contact intimate. These various calculations cannot be applied directly to the situation encountered in electron microscopy since they make many assumptions which, while legitimate for conventional methods and fast beta particles, become invalid with low energy beta particles and layers of emulsion whose thickness is comparable to the size of the grain. In particular, the range limitations of the β-particles cannot be ignored and the emulsion cannot be treated as a continuous medium.

In this article we shall derive a theoretical expression for the expected distribution of exposed grains around a point source of tritium, calculate from this the distribution for a small extended source, and verify this distribution experimentally. The derivation of a general expression, valid for all cases, would present forbidding mathematical difficulties and would be of dubious value since many parameters, such as the range of low energy electrons or the distribution of silver halide crystals in the applied emulsion, are not precisely determined. We have, instead, tried to get an estimate of the expected distribution of grains, using approximations and numerical or graphic methods of integration whenever convenient, for the well defined situation of a point source of tritium situated in a methacrylate section at a certain distance from a tightly packed monolayer of silver halide crystals. This approximates the situation encountered in the electron microscopic autoradiography of thin sections, using the methods described in the preceding article (15). We hope thus (*a*) to define approximately the present limits of resolution, (*b*) to verify this estimate experimentally, and (*c*) to obtain an indication of the important factors influencing resolution.

Three elements of uncertainty combine to limit resolution. The first is the relation between the source of beta particles and the crystals hit by these particles. The second is the relation between the passage of an electron through a crystal and the position of the latent image formed. The third is the relation between the final image observed—a deposit of silver of variable size and shape—and the latent image that produced it. We have seen, in the preceding article, that by using a special fine grain developer the position of a given developed latent image could be located with great accuracy. We have also seen that it was not possible to determine from this the position of the center of the corresponding crystal of silver halide with an accuracy better than 500 A. This last degree of uncertainty is probably beyond our control and can only be decreased by reducing the size of the crystals. We shall therefore study the first part of the process: the probable position with respect to the source of the centers of exposed crystals.

1) *Distribution of Exposed Crystals around a Point Source*

a) General Considerations

The situation to be investigated is shown in diagrammatic form in Fig. 2. Consider a point source placed, in methacrylate of density 1.1, 500 A below an emulsion composed of a single layer of uniform silver halide crystals 0.1 μ in diameter. We assume that the crystals form a perfect hexagonal packing and occupy 57 per cent of the space in a layer 0.1 μ thick. We assume also that the dry gelatin has a density similar to that of methacrylate; the electrons travel therefore, until they hit a crystal, in a continuous medium. The position of a silver halide crystal is defined by the projection of its center on a plane of reference parallel to that of the specimen, and the position of a point is, of course, the projection of that point. When we talk about distribution of

grains, the distance between the source and a crystal is the distance between their respective positions on the plane of reference. If, however, we are considering the path of an electron, this distance is the actual distance between the source and the surface of the crystal. Two main considerations will influence the final results: the range distribution of the emitted beta particles and the geometry of the preparation.

b) Range Distribution of Beta Particles from Tritium

In evaluating the range of a beta particle of given energy we shall neglect the amount of energy needed to produce a latent image. The minimum energy needed is of the order of 100 ev (32) and therefore too small to modify the range distribution curve significantly. The electrons emitted in the decay of tritium atoms have energies varying from 0 to 18 kev. The energy spectrum for tritium is well established (17). It is presented in a convenient form in an article by Robertson and Hughes (18). D. E. Lea (19) has given values, calculated from the Bethe-Bloch formulation, for the range of electrons in material of density 1.0. We have extrapolated these values to the density of methacrylate (1.1) and corrected them for the curved path of electrons, according to Williams (20), to give the foil range. Such calculated values are subject to great possibilities of error (21) for slow electrons. Yet available experimental evidences show them to be, at least, reasonable approximations (22, 23, John W. Preiss, personal communication). Using this information we have plotted in Fig. 1 the distribution of ranges in methacrylate for the beta particles emitted by tritium. This distribution would be very similar for tissue or for paraffin since the densities of these media are not very different. One fact emerges clearly from such elementary considerations: if a source of tritium is placed 1 micron away from the emulsion only 20 per cent of the emitted betas will reach the emulsion; at 2 microns the proportion becomes 2 per cent. This means that in a conventional autoradiograph of a normal histological section, 5 microns thick, for example, the autoradiographic image is produced by a very thin upper layer and therefore represents only a minute fraction of the structures seen in the microscope. This obvious source of artefacts and misinterpretation is considerably reduced when thin sections are used in light microscope preparation (15) and almost completely eliminated at the electron microscope level, with sections thinner than 1000 A.

c) Geometry

In Fig. 2 the source, at *Ym*, is placed arbitrarily 500 A away from the emulsion. The probability that a crystal, placed at a certain distance from the origin, will be hit depends (*a*) on the solid angle from which it is seen by the source and (*b*) on the density of the material through which the electron travels before reaching the crystal.

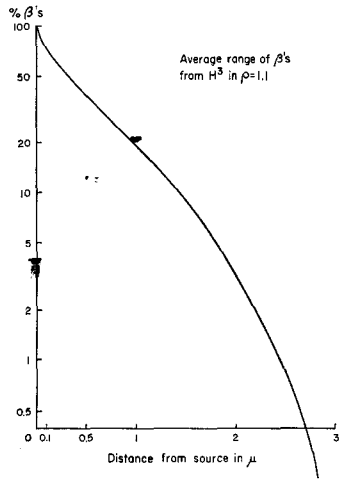


Figure 1

Expected distribution of ranges of β-particles from tritium in a medium of density 1.1 (methacrylate). This distribution is based on the energy spectrum of tritium decays and on the calculated range of electrons, corrected for the curvature of the path.

SOLID ANGLE: Simple geometric considerations give the value of the solid angle at 1-cos θ where θ is the half-angle from which the source sees the crystal (for example $e = \frac{\alpha_1}{2}$, for grain 1 in Fig. 2). As the distance from the source increases the expression for the solid angle can be simplified to $\frac{\pi r^2}{x^2}$ where x is the distance from the crystal to the origin and r is the radius of the crystal (0.05 μ). Thus the solid angle decreases approximately as the square of the distance from the origin.

DENSITY EFFECT: Because of the low density of air any electron leaving the surface of the preparation and being scattered back will probably appear as background, at a considerable

distance from the source. Therefore we can consider that, in order to reach a given crystal, an electron can only travel through methacrylate (density 1.1) or silver halides (density 6.47 for silver bromide). We have found experimentally (15) that the probability that a tritium decay will expose several grains in a monolayer is extremely small. It can be concluded therefore that, because of the relatively high density of silver halides, the passage through a crystal will result either in absorption or in scattering of the particle out of the plane of the emulsion, with a probability close to one. This means that, effectively, crystals which are some distance away from the source will be partially shielded by crystals which are closer. For example, if we assume that, in the diagram of Fig. 2, crystals 1 and 2 are in line with the source, the effective cross-section of crystal 2 will be its total cross-section decreased by the projection of crystal 1. This effect causes a very rapid decrease of the effective cross-section as we go away from the source and is probably an important contribution to resolution. It is clear that this effect will not operate if the emulsion is either very thick or very dilute. In the first case a scattered electron can still hit a crystal in another layer, while in the other case the crystals are too dispersed to shield each other. We found experimentally that, in either case, a loss of resolution was experienced.

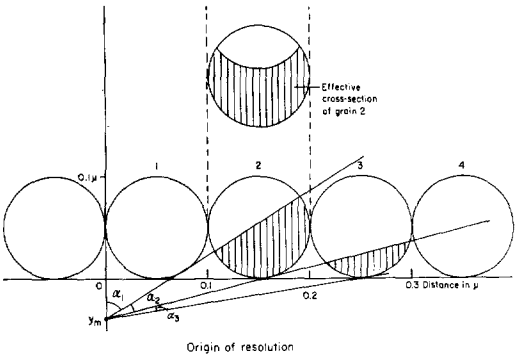


FIGURE 2

Diagrammatic representation of the situation investigated in the resolution calculations given in the text. A point source of tritium is placed at *Ym*, 500 A away from a monolayer of silver halide crystals 0.1 μ in diameter. The effective cross-section of a crystal is decreased by the presence, in the path of the electrons emitted, of other crystals. For example, if grain *2* is immediately behind grain *1*, its cross-section will be decreased by the projected shadow of grain *1*. (See text.)

The extent of this shielding effect was estimated, for a crystal at a given distance from the source, by measuring graphically its effective cross-section, assuming that another crystal is placed immediately in front of it and correcting for the probability, evaluated from the geometry of a packed monolayer of spheres, that such a crystal will be there. As shown in Fig. 3, this relative target size decreases rapidly during the first 0.3 μ, then more slowly for longer distances.

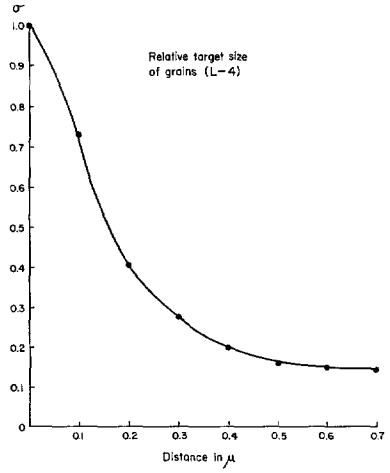


FIGURE 3

Relative target size (or effective cross-section) of grains at various distance from the source. The change in cross-section reflects the shielding provided by grains which are closer to the source, as discussed in the text and in Fig. 2. This curve is valid for a source placed at 500 A and grains having 1000 A diameter only. It was evaluated graphically.

d) PREDICTED RESOLUTION

The density of exposed grains around the point source can be equated to the probability that a crystal, at a certain distance from the origin, will be hit. This, in turn, will be a function of the product of (*a*) the probability that a beta particle will have enough energy to reach the target, (*b*) the relative target size, as defined above, and (*c*) the solid angle. The combined results, expressed as relative grain density around the point source versus distance from the origin, are plotted on a logarithmic scale in Fig. 4. It appears as the sum of two exponential functions, but for all practical purposes can be expressed by the simple relation

$D = D_o e^{-1.6x}$, where D is the density of exposed grains, D_o the density at the origin, and x the distance from the origin in units of $\frac{1}{10}$ micron.

A prediction of the resolution obtained depends largely on our definition of resolution. If we use a formulation similar to the Rayleigh criterion of classical optics (24) the value will be approximately twice the distance at which the density decreases to 50 per cent of its maximum value, *i.e.*, 860 A. The same value is obtained from the definition proposed by Doniach and Pelc (11). If we use the criterion proposed by Gomberg and Schlesinger (14), *i.e.* that two points are resolved if the film density between them falls to one-half the density on each point, we predict a resolution of approximately 1500 A. These definitions are all arbitrary but in this discussion we shall use the first formulation which has the advantage of analogy with the more familiar concept of resolution in optical systems.

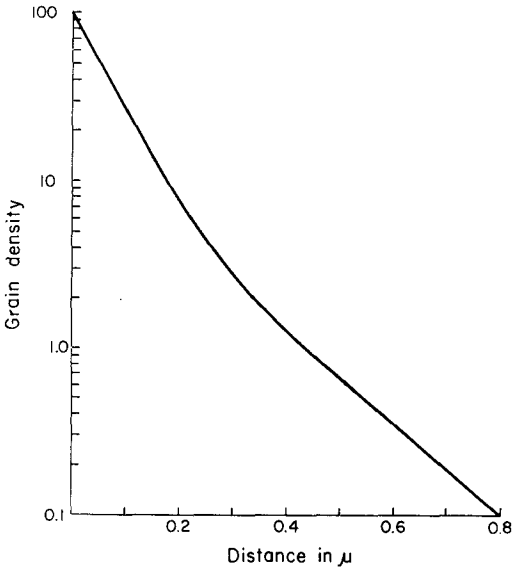


FIGURE 4

Expected density of grains, as a function of distance from the origin, for a point source 500 A away from the emulsion (grain diameter 1000 A). The probability that a crystal will be hit falls to less than 30 per cent of its value at the origin within 0.1 μ and to less than 10 per cent within 0.2 μ. The density distribution curve appears as the sum of two exponential functions but can be approximated by the simple relation $D = D_o e^{-1.6x}$ where x is the distance from the origin in units of 0.1 μ.

A difficulty arises from the fact that the resolution predicted is of the same order of magnitude as the size of the silver halide crystals, but before discussing this point we shall consider some experimental results.

2) *Experimental Verification*

We have used T-2 bacteriophages labeled with thymidine-H^3 [1] as an approximation for a point source. In order to check the point source distribution of Fig. 4 we shall calculate from it the expected distribution of grains around bacteriophages and compare the result to an experimental distribution obtained from electron microscopic autoradiographs of the labeled bacteriophages.

a) Calculated Distribution

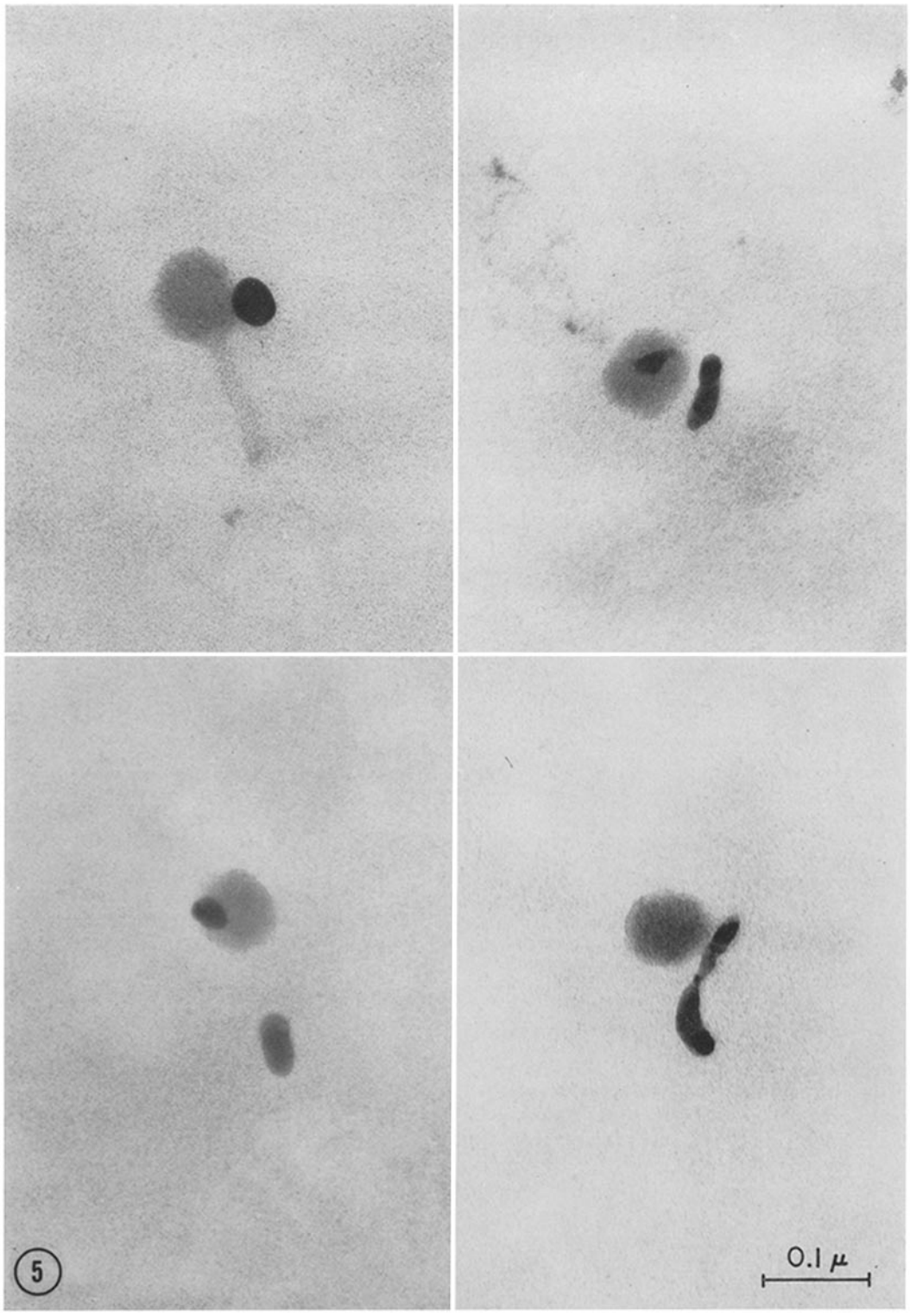
We can approximate the source of tritium (head of the phage) to a sphere, roughly 600 A in diameter (25). Since this diameter is fairly large with respect to the resolution expected we cannot assume that it is a point source. The exponential point source function describing the probability of exposing a grain is therefore integrated over the volume of the phage. This can be done by numerical methods with an accuracy sufficient for our purpose. (The sphere equivalent to a phage head is divided into a number of sectors and, for each point on the x axis, marking the possible position of a silver halide crystal, the contribution from each sector to the probability of a hit is calculated in function of its average distance from the crystal, the distribution function for a point source shown in Fig. 4 and the volume of the sector.)

It is not usually convenient to plot the distribution of grains in terms of density, as in Fig. 4. One might rather plot the radial distribution of exposed grains, *i.e.*, the total number of exposed grains found at distances $x + \Delta x$ of the origin. To calculate such a distribution from the density distribution we divided the plane into concentric circular zones, with equal increments of radius, around the origin and multiplied the density value for a given point by the area of the zone including it (this being proportional to the probability of finding a crystal at that distance from the origin). The resulting distribution normalized to the experimental grain count is shown in Fig. 6 as a continuous line.

b) Experimental Distribution

The bacteriophage preparations are made by the agar collodion filtration technique described

[1] The labeled bacteriophages were kindly provided by Dr. Phyllis Kahn, to whom we are indebted.



by Kellenberger and Arber (26): a drop of the phage suspension is spread over a thin collodion membrane coating a 2 per cent agar surface, the particles are fixed by a brief exposure to OsO_4 vapors and the preparation is placed in a Petri dish until complete diffusion of the liquid into the agar. The collodion film is then floated on a water surface, specimen grids are placed on top of it (on the phage side of the film), and the film is picked up with a square of copper mesh lowered on its upper surface. An emulsion is placed on each screen by the loop method described in the first paper (15). The final preparation consists therefore of a monolayer of silver halide crystals separated from the phage particles by a thin collodion membrane. This membrane has approximately the same electron scattering properties, judged from its apparent density in the electron microscope, as a very thin Epon or Araldite section and is probably of comparable thickness, *i.e.*, approximately 500 A (25, 30). The exposure is calculated to give an average grain count per phage slightly lower than one. After exposure the preparations are developed in the physical developer previously described (15). The bacteriophages are then stained for 20 minutes in 2 per cent uranyl acetate. Photographs of all phage particles (Fig. 5) having at least one associated grain are taken and the distance from the grain to the center of the phage measured. As explained in the previous paper the grains are either small and spherical or larger and comma-shaped. In the first case the center of the grain and in the second case the pointed end of the comma were used to indicate the position of the corresponding latent image. The resulting distribution is shown on the histogram of Fig. 6. It is found to coincide fairly well with the expected distribution.

We find therefore that an experimental distribution of grains around phage particles follows closely the expected distribution of exposed crystals. This expected distribution was, in turn, calculated from a theoretical point source distribution. This gives therefore an experimental verification of the theoretical distribution and a justification of the resolution estimated from it.

It might be of some practical interest to determine the distribution of grains over more extended areas. To this end we have used thin sections of *Bacillus subtilis* labeled with thymidine-H^3 (15), and measured the distribution of grains on either

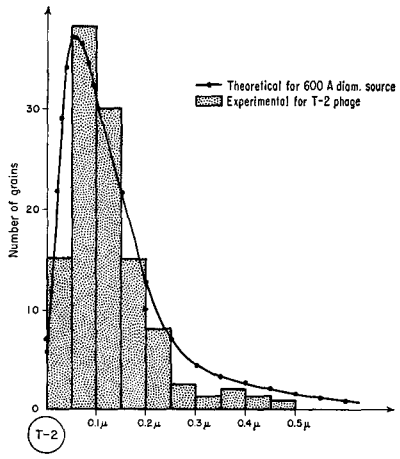


FIGURE 6

Radial distribution of grains around tritium-labeled bacteriophages. The theoretical curve was calculated by graphic integration ot the point source distribution shown in Fig. 4 over a sphere 600 A in diameter. The experimental distribution was measured on 100 photographs such as those shown in Fig. 5. This plot represents the total number of grains (not the density) found at a given distance from the origin.

side of the edge of the nuclear region on cross-sections of the cells (Fig. 7). The resulting distribution is shown in Fig. 8. It is clear that if we had two regions of similar size separated by a distance of 0.1 μ they would be resolved autoradiographically.

DISCUSSION

We might consider first the effect of grain size on resolution. We have seen that if an electron hits a crystal the latent image can appear anywhere on the surface of this crystal and can be therefore as

FIGURE 5

Autoradiographs of T-2 bacteriophages labeled with thymidine-H^3. The fine grain, "physical" developer described in reference 15 was used. The phages were stained with uranyl acetate (only the head of the phage, containing the nucleic acid, is normally stained, but occasionally the tail can be made visible, as in the upper left). × 160,000.

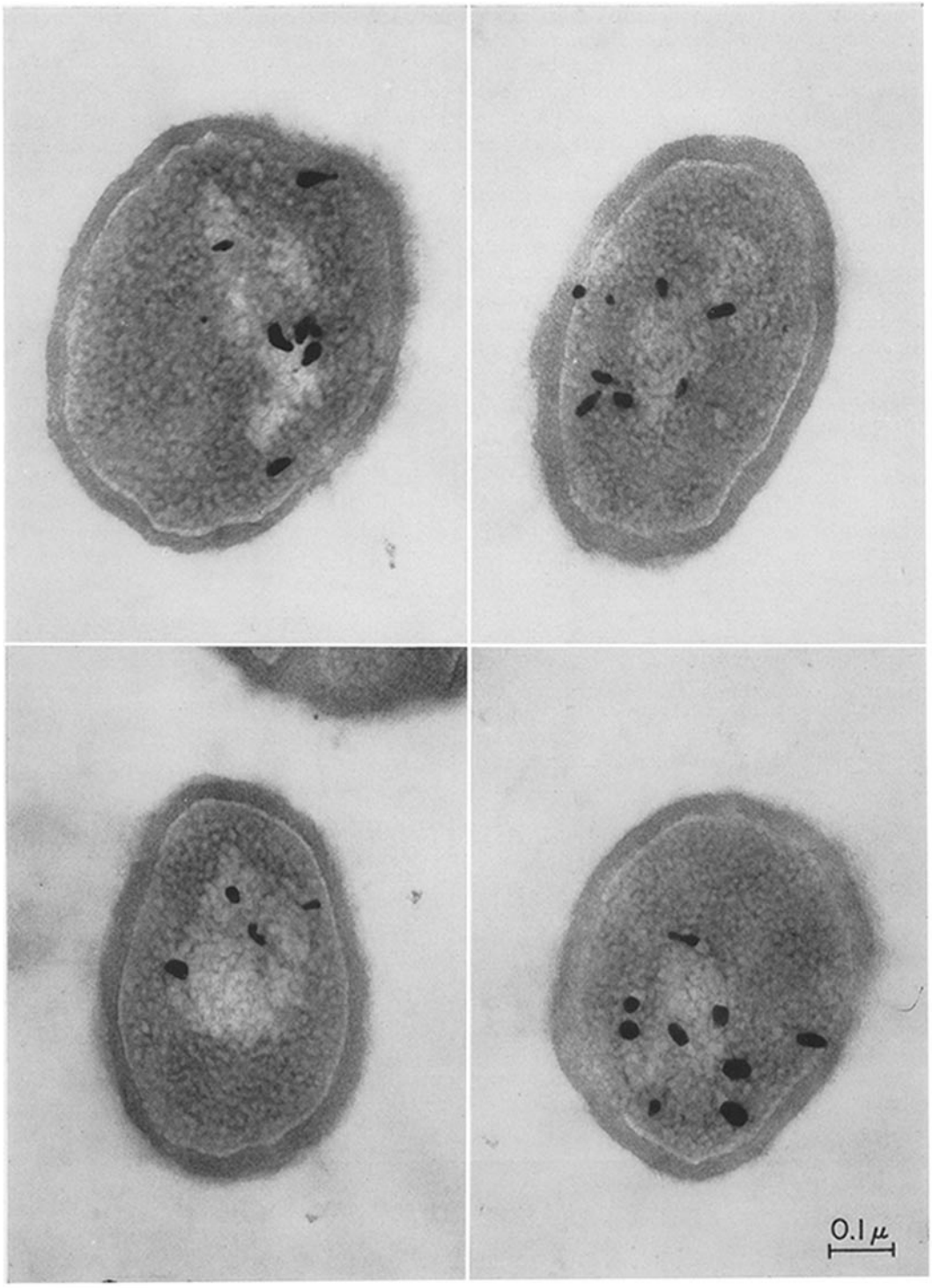


FIGURE 7

Autoradiographs of thin cross-sections of *B. subtilis* labeled with thymidine-H^3. Fine grain development (15). Although many grains are found outside the nuclear region, almost all of them occur very close to it. × 80,000.

far as 500 A away from the center. This introduces an additional uncertainty into our distribution, which was based on the expected position of the center of exposed crystals. In an experiment such as the one with the bacteriophages described above, where the exposure was calculated to produce a very small number of grains for each source, we do not expect that the distribution over a large number of sources will be affected greatly. If we consider, for example, an exposed crystal whose center is 1000 A away from the source, it is clear that the probability that the latent image will appear closer than 1000 A to the source is almost

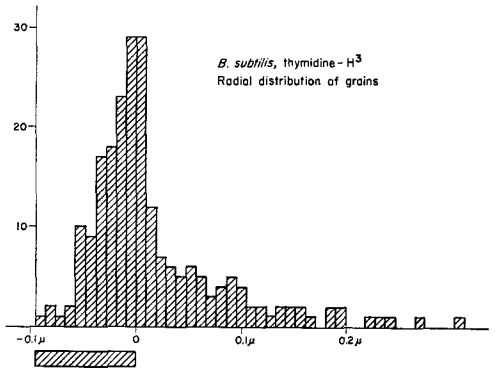


FIGURE 8

Distribution of grains over the nuclear regions in thin cross-sections of *B. subtilis* such as those shown in Fig. 7. Distances were measured with respect to the edge of the nuclear region, the counts on the left of the origin representing counts over the nuclear region. As might be expected, because of increasing areas, the highest probability of a grain occurs at the edge of the nuclear region. Approximately 89 per cent of 220 grains counted were found within 0.1 μ of the nuclear region or over it.

equal to that of its appearance farther away. It is only when we come very close to the source that this situation changes significantly. Therefore, without going into a detailed analysis, we might expect a change on the shape of the density distribution curve only near the origin, very little change after 1000 A, and only a slight over-all effect on resolution. This conclusion is supported by the experimental distribution which fits clearly the predicted distribution for the centers of crystals. It is also confirmed at the light microscope level and on a different scale by the results of S. Bleecken (31).

The crystal size will, however, clearly affect resolution in cases when the number of grains per source must be high. If we want to separate autoradiographically two point sources separated by 1000 A there must be enough grains developed to give a statistical meaning to the concept of grain density. If the diameter of a crystal is 1000 A we can, at most, have two grains between the two sources. It will not be sufficient to define them. If the diameter of the crystals was reduced to 100 A (without changing the shape of the density distribution) it would be possible to have enough grains to define the two sources and the resolution of approximately 1000 A would become again a meaningful concept. Another factor, related to the density of grain counts needed, is that, as the exposure increases, a crystal immediately above a point source might be hit a number of times but still produce only one grain, while grains farther away have an increasing probability of having been hit at least once, thereby decreasing the resolution (11). In conclusion, therefore, if we assume a certain predicted resolution, an emulsion with a crystal size of the same order as the resolution expected will be good enough to separate extended sources (such as the nuclear regions of bacteria considered above) but for smaller sources a finer grained emulsion will be needed.

The density distribution itself will be affected to some extent by the crystal size and also, very markedly, by the distance of the source to the emulsion. We have plotted on Fig. 9 the distributions expected for crystals of 1000 A and 100 A and for distances between source and emulsion of 500 A and 100 A. In the case of thin sections we cannot do much to reduce the specimen to emulsion distance since the thickness of the section determines this. It might be interesting to speculate on the other approach to resolution: the decrease in crystal size in the silver halide emulsion.

On the basis of energy loss relationships Pelc has proposed that 100 A to 500 A would be a limiting crystal size for registering tritium beta particles (8). This estimate seems reasonable since Perfilov *et al.* (27, 28), for example, have described a nuclear emulsion having a grain size of 500 A and having a high sensitivity to electrons at minimum ionization. (The rate of energy-loss with respect to distance for a charged particle decreases with higher energies until a minimum is reached. For electrons this minimum occurs at 1 Mev. An emulsion capable of registering 1 Mev electrons will be sensitive to electrons of any energy.) β-particles with the average energy for tritium (5.7 kev) will have a rate of energy loss approximately 17 times higher than 1 Mev

electrons (21, 29), and therefore a much higher probability of being registered in a given emulsion (2). A decrease in the size of the crystals seems therefore to provide a promising approach toward improving resolution to some extent, although this will be limited by the size of a silver halide crystal still capable of registering one decay (8).

We have reached therefore the following conclusions: on the basis of the geometry of the preparation and the range distribution of tritium particles, we can predict a resolution of the order of 0.1 micron when the methods of autoradiography outlined in the preceding paper are used. The calculated distribution of exposed grains leading to this prediction has been verified experimentally using bacteriophages as a test specimen. When emulsions having grains with a diameter of 0.1 micron are used, the number of grains per unit area proves to be a practical limit to the resolution if small sources are to be separated. With extended sources, of simple geometry, the resolution achieved comes close to the predicted limit. It seems therefore that, using the present methods on tissue sections, we can expect a limit in resolution of the order of 0.1 μ. A significant improvement of this limit will require a considerable decrease both in the specimen thickness and in the size of silver halide crystals.

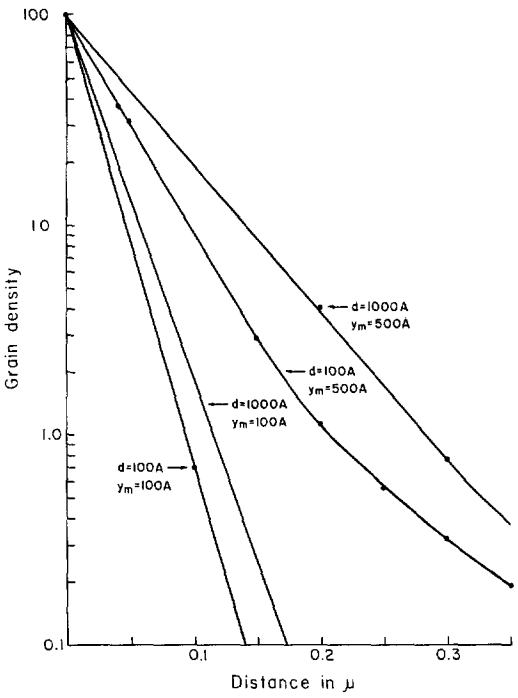


FIGURE 9

Expected grain density distributions, calculated as explained in the text for Fig. 4, for a variety of situations: (*a*) Crystal diameter 1000 A, source 500 A from emulsion; (*b*) Crystal diameter 100 A, source 500 A from emulsion; (*c*) Crystal diameter 1000 A, source 100 A from emulsion; (*d*) Crystal diameter 100 A, source 100 A from emulsion.

It is seen that resolution will increase if either the diameter of the silver halide crystals or the thickness of the specimen is decreased. For reasons mentioned in the discussion, a decrease in crystal diameter would have a more important effect on the resolution than would appear from this graph.

From a practical point of view we reach the following conclusions: if the problem investigated requires the identification of sources of various sizes separated by relatively great distances, very satisfactory results can be expected, the grains being found over or very close to the sources. Such a situation was found, for example, in the case of labeled zymogen granules in pancreatic exocrine cells (33). A good discrimination of the grain counts will also be obtained with extended sources separated by at least 1000 A. If, however, closely spaced small sources are to be separated, finer grained emulsions will be needed. In any case it is clear that the present techniques are sufficient for their application to a large number of problems at the subcellular level.

In addition to providing high autoradiographic resolution, the electron microscopic method has some considerable advantages over the conventional methods. The optical resolution of the microscope allows us to see and recognize clearly the labeled structure, even if it is beyond the resolving power of the light microscope (as in the case of bacteriophages, for example), to see photographic grains much below this resolving power and to measure with great accuracy the distance between the grain and the structure. In addition, the great depth of focus of the electron microscope permits us to obtain a complete photographic record of both grains and structures. For these reasons accurate grain counts can be performed and, as we have seen in the first paper, relative quantitation is obtained with good reproducibility. With some improvement in methodology, absolute quantitation is not beyond the scope of this technique. In spite of some drawbacks, the major one being its low sensitivity, the technique of electron microscopic autoradiography should therefore prove itself useful in cellular biology.

This work was supported by a grant from the National Science Foundation.

Received for publication, June 14, 1962.

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