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Recent developments in the use of autoradiographic techniques with electron microscopy

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[Plate 32]

The development of techniques for observing tissues by electron microscopy has opened a whole new world of structure to the biologist. As the descriptive detail of cell organization has built up, the pressure for methods that would link these new structures to the biochemistry of the cell has also increased. It was inevitable that an attempt to apply the radio-isotopic tracer experiment at the electron microscope level should be made: it was equally certain that this attempt would involve autoradiography, since the nuclear emulsion is the only detector for radio-isotopes with powers of resolution anywhere near sufficient for the size of organelle under study.

In attempting to marry the two approaches of electron microscopy and autoradiography, the logical starting-point has been to take the established product of the one technique—the conventional thin section of tissue—and attempt to adapt the methods and materials of autoradiography to it. To this day, the majority of publications in this field deal with material fixed in aldehydes, postfixed in osmium tetroxide, embedded in epon or araldite, and sectioned at 50 to 100 nm. Most of the information available deals with this type of specimen as the source of radioactivity.

Resolution and efficiency

In an e.m. autoradiograph, the tissue section is covered with a very thin layer of nuclear emulsion—usually a single layer of silver halide crystals. Some of the β particles leaving the section travel into or through the emulsion monolayer, losing energy as they do so. This energy loss, if it occurs within a crystal of silver halide, may create a latent image in that crystal, which becomes converted into a coiled ribbon of metallic silver by the standard process of chemical development (figure 1*a*, plate 32).

The emulsion most frequently used, to judge from published accounts, is Ilford L4, in which the mean diameter of the crystals is about 140 nm. After development with Microdol X, silver grains are produced 200 to 300 nm in diameter.

Three factors combine to make it difficult to identify the origin in the section of the β particle which produced the grain.

(i) The developed silver grain is larger than many of the underlying structures, and much larger than the latent image speck which preceded it. It is very difficult to determine the precise position of the latent image speck from examining the grain.

(ii) Even if one could specify the position of the latent image, this is not necessarily on the path of the β particle that caused it. The latent image forms somewhere within a silver halide crystal 140 nm in diameter, at a point determined during manufacture of the emulsion. There is no certainty that the developed filament of silver occupies the same position as the parent crystal.

(iii) The third factor is that β particles may be emitted from a labelled source in any

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direction, over a spectrum of initial energies, so that crystals at a distance from the source may be hit (figure 1*b*).

The techniques of preparing e.m. autoradiographs have evolved as attempts to minimize each of these sources of error. The centre of the developed grain will be nearer the position of the latent image if the size of the grain is kept as small as possible. The latent image will be on average nearer the path of the β particle if the size of the silver halide crystal is as small as possible. The scatter of hit crystals about a labelled source will be minimized with isotopes, such as tritium, which have a very low maximum energy. In addition, the section and emulsion layers should be as thin as possible, and in contact.

Several adequate methods exist of applying a thin, uniform layer of emulsion to a thin section. Caro's method, in which a loop of wire is used to pick up a bubble-like film of emulsion (Caro & van Tubergen 1962; Caro 1969) is the one most frequently quoted in the literature. My own preference is for the dipping technique as described by Salpeter & Bachmann (1964), in which the monolayer is formed on the flat substrate provided by a microscope slide, rather than on the irregular profile of a support grid. Both methods are capable of producing reasonably uniform monolayers of silver halide crystals over the specimen. These techniques are not new, and they have been fully discussed in the literature, together with appropriate methods of processing the emulsion and staining the specimen.

We are unfortunately limited in our progress towards thinner and thinner sections, smaller crystals and smaller developed grains by the overall efficiency of the system in detecting β particles. This can be seen from some simple calculations, based on a tissue containing tritium at a level of 1 μCi/g. This, incidentally, is already a high level of activity. Workers using tritiated thymidine regard 1 μCi/g body mass as about the maximum that can be *injected* into an animal if radiation damage to dividing cells is to be kept within reasonable limits. Clearly, to get 1 μCi/g in the fixed and embedded tissue will usually need far higher doses than this. I have assumed that a section 50 nm thick has been coated with a monolayer of Ilford L4, and developed to give an overall efficiency of 10 %—i.e. each grain represents 10 disintegrations in the source (Bachmann & Salpeter 1967). After 6 months exposure, 1 grid square, containing 8000 μm^2 of section, would have over it 23 developed grains on average. This is a reasonable figure, particularly if the grains are associated with one or two organelles, forming a relatively small percentage of the tissue.

Now let us assume that an improved method of autoradiography permits us to handle sections cut reproducibly at 10 nm, and coat them with a monolayer of crystals of the order of 20 nm in diameter. Such a method would give a significant reduction in our errors of resolution. But a section 10 nm thick would only have one-fifth the radioactivity of the 50 nm section; and a beta particle requires a certain minimum length of track within a silver halide crystal in order to produce a developable latent image. Such a reduction in crystal size would greatly increase the probability of β particles travelling through the emulsion layer without leaving latent images to mark their passage. If we assume an efficiency of 1 % for this new emulsion, we now find that, after 12 months' exposure, we have an average of one developed grain per grid square.

Advances in emulsion manufacture and in e.m. techniques may well give us useful improvements in efficiency, in resolution, or in reproducibility. It is difficult to see how any refinement of the present system can produce really significant gains in all three simultaneously. In short, we are stuck with a system in which the resolution of the autoradiograph is worse than the resolution of the electron microscope by a factor of 50 to 100.

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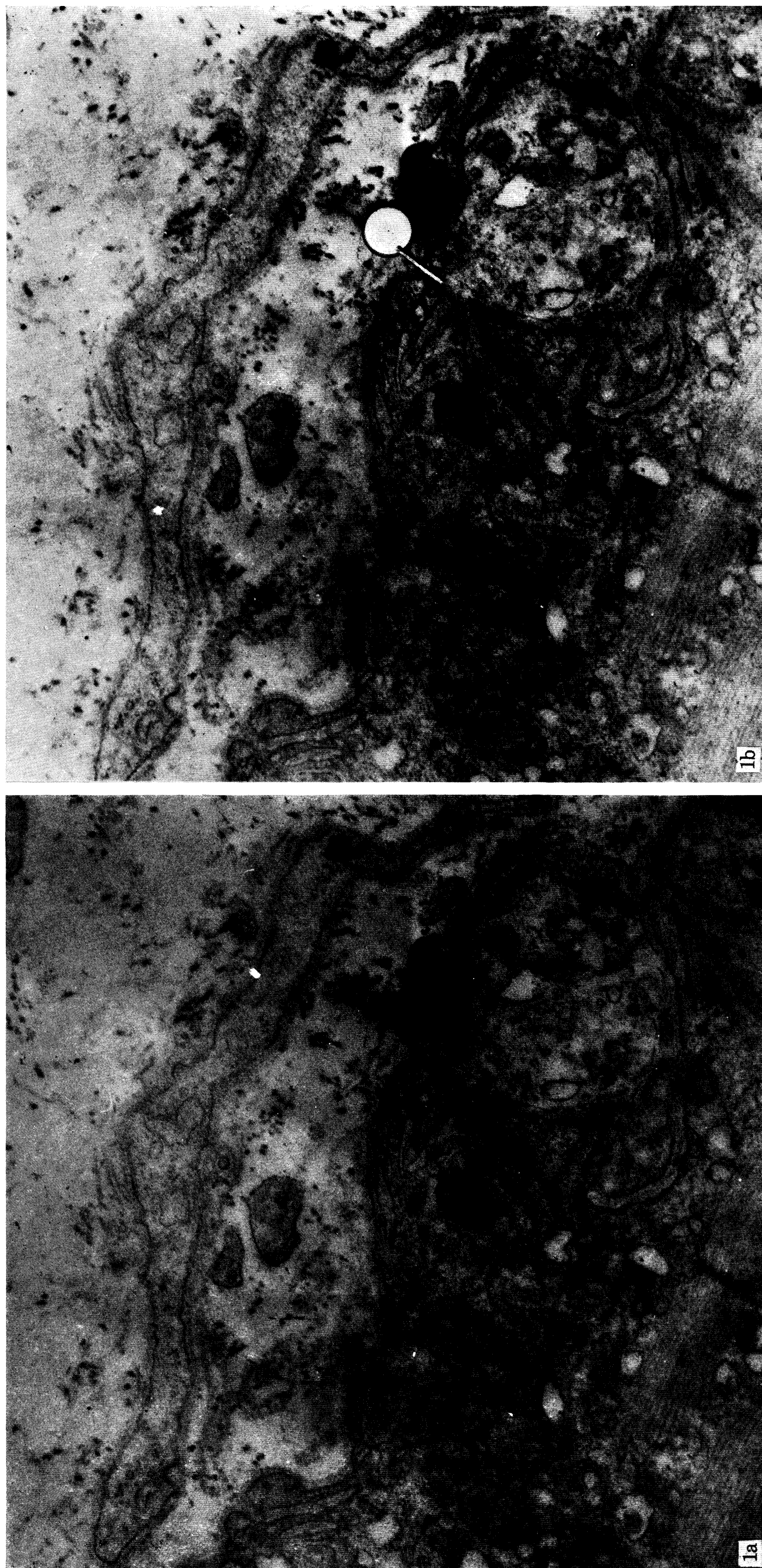

FIGURE 1. (*a*) E.m. autoradiograph of a neuromuscular junction after *in vitro* treatment with [^{3}H]DFP. Ilford L4 emulsion with Microdol X development. Note the large size of the developed grain. (*b*) the same micrograph with hypothetical position of parent crystal and β track. Autoradiograph prepared by Dr M. M. Salpeter.

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Setting up a valid technique

Where does this leave the aspiring autoradiographer? His major problem will be studying the distribution of silver grains over his specimens, to try and deduce which tissue components are labelled. Since the resolution of this system (the pattern and dimensions of the scatter of grains around each source) is central to any attempt to analyse the observed grain distribution, factors which might influence the resolution in the preparation of his material must be very carefully controlled. The thickness of his sections must be uniform. This is usually judged by their interference colour as they float in water (Peachey 1958; Bachmann & Sitte 1958). But there may be differences between individuals in the accuracy with which they assess interference colour (Williams & Meek 1966). As a control measure, the careful autoradiographer will calibrate his own performance against measurements with the interference microscope (Salpeter, Bachmann & Salpeter 1969). Similarly, repeated checks should be made to be certain that uniform layers of silver halide crystals, closely packed but not more than one crystal thick, are being used.

To these controls, I should like to suggest the addition of one more, to demonstrate that the conditions of exposure are not resulting in the loss of latent images after their initial formation. The appropriate control (too seldom applied at the light microscope level, unfortunately) is to fog one experimental slide with light or radiation, before putting it for exposure and development with the rest of the series. We have found that semi-thin sections of tissues fixed in gluteraldehyde and postfixed in osmium tetroxide may cause dramatic loss of latent images over the section. Salpeter & Bachmann (1964) have observed the same effect with e.m. autoradiographs, and have recommended covering the section with a 5 to 6 nm layer of carbon before applying the emulsion.

It is easy to assume that the same precaution will prevent latent image fading in every case. My own experiences with this artefact at the light microscope level suggest that this is a dangerous attitude. Fading is favoured by the presence of moisture and of oxidizing agents in the emulsion (see, for instance, Rogers 1967). Careful drying of the emulsion and replacement of air by an inert gas in the exposure box are essential steps if latent images are to survive throughout an exposure of several months. Even the choice of a developing agent can affect the severity of fading (Rogers & John 1969), and some of the development routines that have been suggested in the attempt to produce small grains for electron microscopy would seem quite likely to be very sensitive to this artefact.

Since these factors influencing the severity of latent image loss may well vary from one laboratory to the next, it seems reasonable to expect an investigator to demonstrate that he has controlled this artefect in his experimental material. Fading, if it is associated with some factor in the specimen (such as osmium, perhaps), can be anatomical in its distribution, distorting the grain distributions in a non-random fashion.

The distribution of silver grains around model sources

Let us assume that a technically satisfactory series of e.m. autoradiographs is available for analysis. This analysis must be based on the patterns of distribution of silver grains around sources of various shapes and sizes, in the conditions of that particular experiment. Until recently, the only guidance on the likely scatter of grains about a labelled source were several

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theoretical treatments of point sources under various conditions of section and emulsion thickness (Caro 1962; Pelc 1963; Bachmann & Salpeter 1965), and one experimentally obtained grain distribution for one set of conditions (Caro & Schnös 1965).

When one examines the many simplifying assumptions necessarily made in the theoretical papers, and the effects that differences in techniques, and in the size and shape of labelled sources, are likely to have on the grain distributions, this body of evidence is clearly insufficient as a firm foundation for rigorous analysis of autoradiographs.

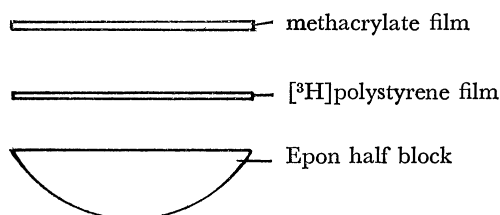

FIGURE 2. Diagram to illustrate the [^{3}H]polystyrene film used as a linear source by Salpeter *et al.* (1969).

Recent work from Salpeter and her colleagues (Salpeter *et al.* 1969) has transformed the position. They began by making a series of well-defined linear sources. A thin film of [^{3}H]polystyrene was formed, about 30 nm thick. This film was then embedded between Epon and methacrylate to make a composite block (figure 2). The block was sectioned at right angles to the plane of the radioactive polystyrene. Each section therefore included a line of [^{3}H]polystyrene of known thickness, which could be recognized on micrographs.

Taking sections whose thickness had been checked by interferometry to be 45 to 55 nm, autoradiographs were prepared with a monolayer of Ilford L4, developed with Microdol X. On micrographs at constant magnification, over 1000 grains were examined, and the distance from the centre of the grain to the polystyrene line measured. The density of grains was high near the line, falling off with increasing distance from the source, to background levels (figure 3).

Precisely similar experiments were carried out with other section thicknesses, with monolayers and double layers of Kodak NTE emulsion, and with development techniques giving different sizes of developed grains. Grain distributions around the 'hot line' were plotted in each case. As expected, the thinner the section, the less the scatter; the smaller the crystal halide size and the thinner the emulsion layer, the less the scatter; the smaller the size of the developed grain, the less the scatter around the polystyrene line.

The striking observation made by Salpeter *et al.*, the one that opened the way to their subsequent work, and made it of so much value, was that the *shape* of the distribution curve was to all intents identical in every case. The scale on which the distribution curve was measured differed with each combination of variables, but the pattern of scattered grains around the line was constant.

If we now recalibrate the distribution curve in units that are related to the shape of the curve itself, instead of in nanometres, we have a standard curve that fits each of a wide range of experimental methods (figure 4). Salpeter *et al.* chose the half-distance, or h.d., as their unit. This is the distance from the 'hot line' within which half the grains produced by it lie. With L4, Microdol X, and a 50 nm section, the h.d. value is 145 nm. The recalibrated curve is now valid for all their data, though the h.d. values they found ranged from 80 to 165 nm.

Now it is clear that the pattern of distribution of grains varies with the size and shape of the source. The distribution is radially symmetrical about a point source, bilaterally symmetrical

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on either side of a straight line source, and so on. The theoretical function relating the distribution about a line to that about a point can be calculated. So, using the generalized curve for a straight line as their starting-point, Salpeter *et al.* (1969) computed the shape and

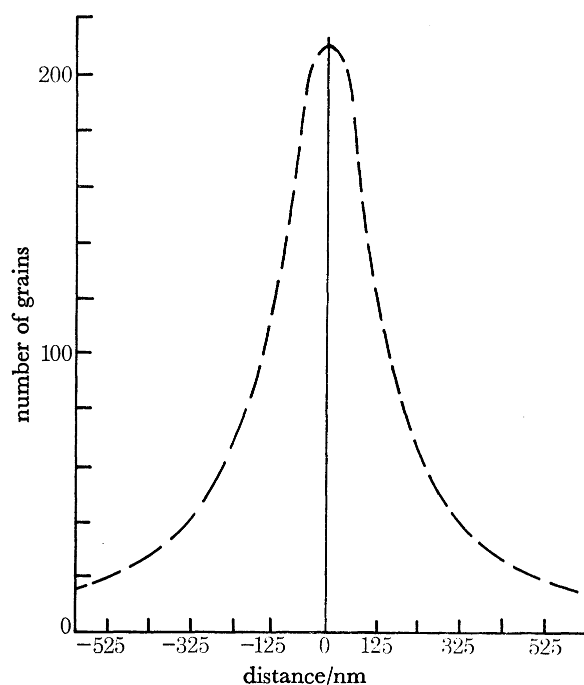

FIGURE 3. The distribution of the centres of developed silver grains relative to a linear radioactive source: Ilford L4 emulsion developed with Microdol X. Modified from Salpeter *et al.* (1969).

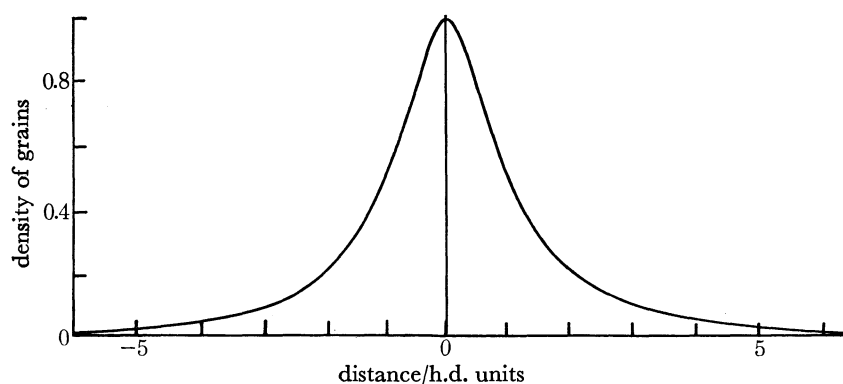

FIGURE 4. The distribution of the centres of developed grains relative to a linear radioactive source: a generalized curve. Modified from Salpeter *et al.* (1969).

dimensions of the distribution of grains about a point source, in the same units of h.d. They were able to go further, producing whole series of distributions for hollow and solid circular sources of different radii, and for hollow and solid band sources of different widths. All these are expressed in units of h.d., defined as the distance from a linear source that includes half the grains produced by it.

For example, let us consider a series of autoradiographs of 50 nm sections, coated with a monolayer of L4, and developed with Microdol X. Figure 3 illustrates the curve of distribution of silver grains around a straight line source, giving an h.d. value of 145 nm. The

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distribution around a point source, around the edge of a solid disk of radius 4 h.d., and around a hollow circle of radius 4 h.d., are all shown in figure 5.

The impact of this mass of detailed information on the analysis of autoradiographs is difficult to overestimate. If an experiment with e.m. autoradiography employs one of the combinations

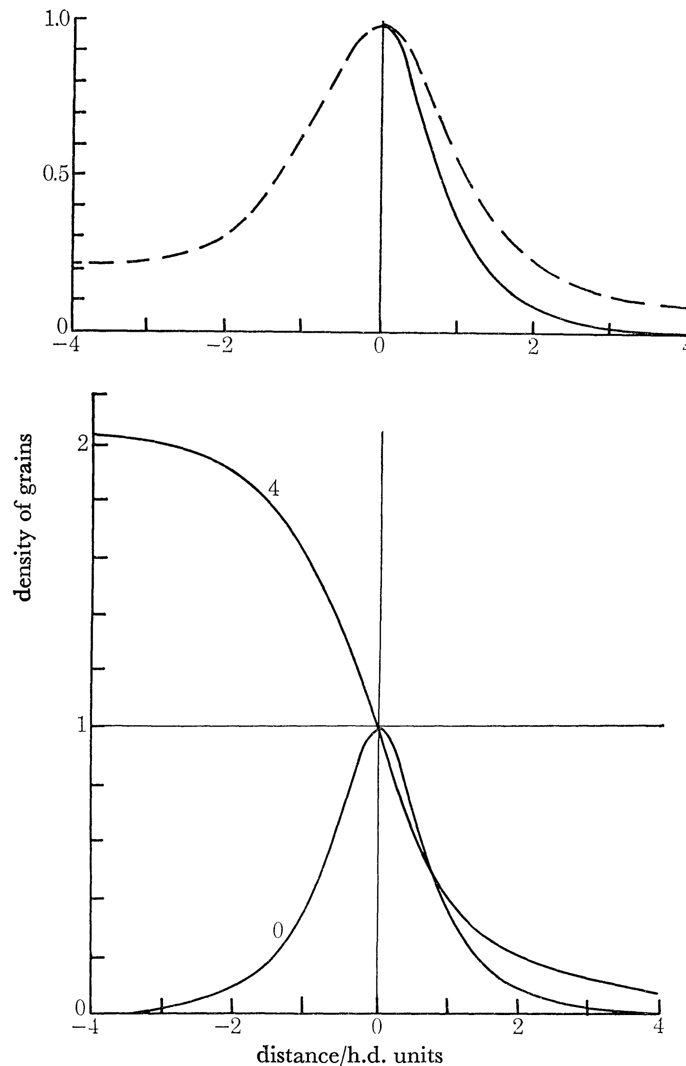

FIGURE 5. (*a*) The distribution of silver grains relative to a point source of radioactivity (solid line) and a hollow circular source of radius 4 h.d. units (broken line). Positive values on the abscissa indicate regions outside the circle: negative values inside the circle. (*b*) The distribution of silver grains relative to a point source of radioactivity (line 0) and to the edge of a solid disk source of radius 4 h.d. units (line 4). Positive values indicate regions outside the disk: negative values regions inside the disk. Data derived from Salpeter *et al.* (1969).

of variables studied by Salpeter *et al.* (1969), the h.d. value is immediately to hand. If the technique is sufficiently different from any of these examined, the h.d. value will have to be measured experimentally with a similar 'hot line'. In either case, a wealth of information becomes available on a wide range of sizes and shapes of source, and the computation of grain distributions for other shapes of source is quite possible.

Without any doubt, this study from Dr Salpeter's laboratory is the most important

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contribution to the field of e.m. autoradiography in recent years, opening the way to precise and quantitative analyses of observed grain distributions.

The analysis of electron microscopy autoradiographs

The simplest and earliest method of analysis was to scan a number of sections, form a subjective opinion about the probable source, and carefully select one or two 'typical fields' for micrographs for publication. This is clearly unacceptable. The possibilities of error are enormous, unless the pattern of labelling is quite unequivocal. Numerical analysis of e.m. autoradiographs began by recording the tissue component underlying the centre of each silver grain, and producing a table of components with a total score of grains opposite each. This is also an inadequate method. It takes no account of the relative volumes of specimen occupied by the various tissue components. If every component is uniformly and randomly labelled, the component with the largest area on the sections will have the most grains over it.

Table 1. A comparison of grain counts with grain densities

	% total grains	grains per unit area
rough e.r.	48	26
Golgi complex	14	30
peripheral cytoplasm	12	12
collagen	16	10

These data are derived from e.m. autoradiographs prepared 1 h after the injection of [^{3}H]proline into guinea-pigs (Ross & Benditt 1965).

Clearly, the distribution of grains over the section must be related to the percentage of the tissue occupied by the various cell components. In such a system, first used by Ross & Benditt (1965) in studying the dynamics of collagen synthesis, two measurements are made for each tissue component—the percentage of the tissue occupied by that component, and the percentage of the total silver grains lying over it. Statistical methods can then show if any component is significantly labelled, and changes in the pattern of labelling with time can be demonstrated.

Methods for measuring the relative tissue volumes of different cell constituents have been well worked out, and are closely associated with the name of Dr Weibel (for a recent review, see Weibel (1969)).

In particular, the methods of tissue sampling which Weibel and his colleagues have developed are of crucial importance to this whole problem of analysis of autoradiographs, since they are designed to avoid the subjective choice of areas to photograph. It is quite possible to distort a random grain distribution into 'significant labelling' by basing an analysis on a series of micrographs taken from areas selected on a subjective basis.

Table 1 assembled from data from Ross & Benditt (1965) shows the grain counts found over four tissue compartments in autoradiographs from animals killed 1 h after the injection of [^{3}H]proline. The percentage of the total number of grains that was found over each compartment would suggest that rough endoplasmic reticulum is far more heavily labelled than any of the others, with collagen the next in order of radioactivity. As soon as corrections for relative volume are made, however, the grain density over the Golgi complex is seen to be higher than that over rough endoplasmic reticulum, while collagen becomes the least heavily labelled compartment.

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But even the method of Ross & Benditt is not getting the best information out of the autoradiographs. In an experiment in which only one cell component is labelled—for instance the rough endoplasmic reticulum—grains will be found over many other cytoplasmic organelles, representing the scattered tail of the distribution curve. If two organelles are often found close to one another, such as mitochondria and infoldings of the cell membrane, labelling confined to one will give a highly significant grain count over the other.

What is needed is a method of analysis which takes into account the distribution of grains about a labelled source as well as the spatial arrangement of tissue components in the section. Dr Williams, of Sheffield, has recently published a discussion of the problems of analysis, and a suggested procedure for getting around many of them (Williams 1969).

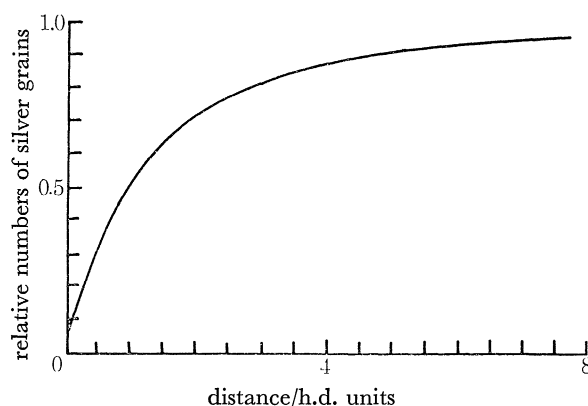

FIGURE 6. The cumulative distribution of grains around a point source of radioactivity. Modified from Salpeter *et al.* (1969).

If we return for a moment to the distribution curves of Salpeter *et al.* (1969) figure 6 illustrates the distribution of grains about a point source. A circle of radius roughly 1.5 h.d. centred on the source will contain half the grains produced by it. Turning this round, a circle of similar radius about the centre of each silver grain will have a 50 % probability of including the source. Williams's analysis is based on these 50 % probability circles. A table is once more constructed of tissue components. Under the heading of 'grains' is scored the number of times that component appears within one of the 50 % probability circles around a developed grain. Next the relative tissue volume occupied by each component is found, by covering the same micrographs with a number of systematically or randomly placed circles of identical size, and recording the frequency with which various components appear within them. A χ^2 test will indicate at once which items, if any, are more, or less, heavily labelled than their volume ratio would lead one to expect.

The choice of tissue components can increase the information given by this analysis. There is no need to restrict this list to single organelles (see table 2). Junctional items can be listed and scored; for instance, circles containing mitochondria in contract with rough endoplasmic reticulum can appear as a separate item. A third group of items Williams calls 'compound'. This is designed to cope with structures which will never be large enough to fill a circle—for instance, nuclear membrane, or plasma membrane. These can only occur together with other items in a circle. But these other structures will also appear in the list of components as single items. A comparison of grain densities over junctional or compound items with that over the

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appropriate structures as single items may indicate that a junctional region or a cell membrane is specifically labelled, even though most of the silver grains lie over the organelles to either side.

Table 2. An example of the listing of cell components for the analysis of e.m. autoradiographs

single items	junctional items	compound items
nucleus	dense bodies/ribosomes	ribosomes/plasma membrane
phagosomes	phagosomes/ribosomes	mitochondria/plasma membrane/ribosomes
mitochondria	nucleus/ribosomes	smooth vesicles/ribosomes
ribosomes	mitochondria/ribosomes	
dense bodies	dense bodies/mitochondria	
	phagosomes/mitochondria	
	mitochondria/ribosomes/nucleus	
	mitochondria/ribosomes/dense bodies	

Measurements of radioactivity from electron microscope autoradiographs

Once one or more organelles are found to be labelled, it is possible to go on and determine their radioactivity from the autoradiographs. Williams (1969) has suggested using the circle data that formed the previous step in the analysis as a basis for doing this. The *relative* activity of each organelle can be expressed directly in terms of grains per unit volume. This figure can be converted into *absolute* terms, if the efficiency of the autoradiographic method is known. Values for the efficiency of various techniques have been published in the literature (Caro & Schnös 1965; Bachmann & Salpeter 1967), but it is better to determine the efficiency of one's own experimental technique, as slight differences in the conditions of development and exposure of the autoradiographs can lead to considerable variation.

If one component alone appears to be heavily labelled, the best approach is to prepare distribution curves of grains around that organelle. On the series of micrographs used for the preceding analysis, the shortest distance from the centre of each developed grain to the edge of the organelle is measured. The distribution histogram so obtained is then compared with the distribution predicted from the work of Salpeter *et al.* (1969) around a simple geometrical source that approximates the organelle in size and shape.

Figure 1, for example, is an autoradiograph of Dr Salpeter's showing a motor end-plate labelled with [^{3}H]DFP. The analysis of a number of these micrographs was carried out (Salpeter 1967), measuring the distance from the centre of each grain to the *presynaptic* membrane. This gave a distribution curve which clearly did not fit the one predicted for a radioactive line in this position (figure 7). A similar distribution curve for grains relative to the *postsynaptic* membrane, however, agreed very well with the predicted curve (figure 8). The advantage of carrying out this extra analytical step is that one not only confirms the identity of the labelled structure, but determines the total number of grains that should be attributed to it with a fair degree of precision, even though many of these may lie over adjacent structures. From this material, Dr Salpeter (1967) was able to calculate the density of DFP-binding sites, and of acetylcholinesterase molecules, on the post synaptic membrane. Her figures were in reasonable agreement with those found by β-track autoradiography and liquid scintillation counting for the number of sites per complete motor endplate (Rogers, Darzynkiewicz, Barnard & Salpeter 1966).

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In many experiments several or even all the tissue components may be labelled, and this detailed distribution-analysis becomes impossibly complicated. Even in this type of experiment, however, by carefully selecting the items in one's list of components to include areas of each organelle relatively far from junctional regions, and comparing them with junctional and compound regions, the analysis can cope with many of the problems of crossfire, caused by the inevitable scatter of silver grains around the labelled source.

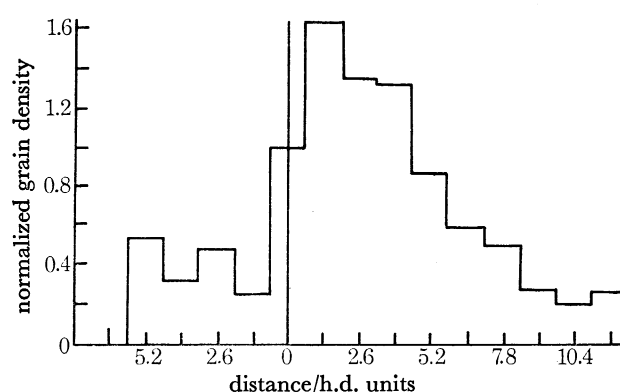

FIGURE 7. The distribution of silver grains about the presynaptic membrane of neuromuscular junctions labelled with [^{3}H]DFP. The line at zero represents the membrane: values to the left lie over the nerve terminal, to the right over the postsynaptic region. Data derived from Salpeter (1967).

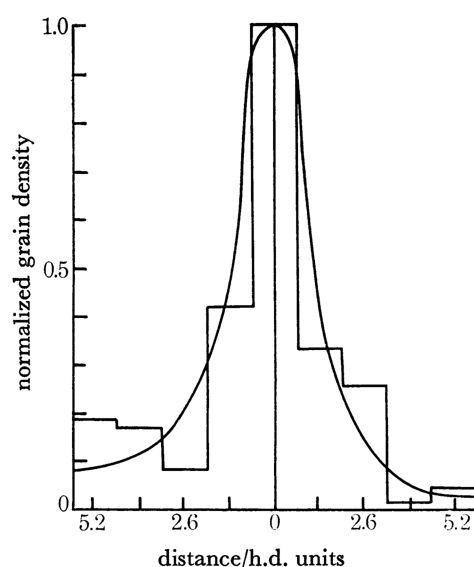

FIGURE 8. The distribution of silver grains about the postsynaptic membrane of neuromuscular junctions labelled with [^{3}H]DFP. The smooth curve represents the distribution predicted for a radioactive line following the course of this membrane: the histogram represents the observed grain distribution. Data derived from Salpeter (1967).

THE COST-EFFECTIVENESS OF ELECTRON MICROSCOPY AUTORADIOGRAPHY

It is one thing to outline a way of analysing autoradiographs; it is quite another to decide when such an analysis is necessary. The autoradiographer must weigh the cost of any projected experiment in money, and, in particular, in time. To perform a satisfactory experiment, capable of reasonable interpretation, the following steps are necessary.

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(i) Control experiments to establish the thickness and uniformity of sections and of emulsion layers.

(ii) Control experiments to establish that exposure and development conditions are not producing latent image loss.

(iii) Exposure periods that will probably be measured in months, even with high levels of injected radioactivity.

(iv) Scanning a series of autoradiographs in order to get the feel of the material, and perhaps setting up a pilot analysis to check that the list of items chosen will give the required information.

(v) Taking a large series of micrographs at constant magnification, from areas of section selected on some carefully predetermined pattern, to avoid subjective bias.

(vi) Determining the h.d. value for the autoradiographs: if this cannot be obtained from the literature, a separate experiment will be needed with a 'hot line' source.

(vii) Analysing the tissues present in 50% probability circles around each silver grain on the micrographs.

(viii) Making a similar analysis with circles distributed randomly over the same prints to find the relative volumes of each tissue component.

(ix) Making some sort of statistical analysis of the data generated.

(x) If the results at this stage indicate that further work would be useful, it may be necessary to approximate the labelled tissue component to a simple shape and size, and re-analyse the micrographs, to produce a distribution curve of grains around the source for comparison with predicted distributions.

Clearly, an effective experiment with e.m. autoradiography is neither easy nor rapid. It cannot be emphasized too much that this is not a survey technique, not a process to be undertaken blithely 'to see if it shows anything'.

On the other hand, as the climax to a series of experiments, in which liquid scintillation counting and light microscope autoradiography have already defined the problem, e.m. autoradiography can provide answers which are not available to us in any other way. The investment in time and work can be well justified if one ends up, as Dr Salpeter did in her study of [^{3}H]DFP at the motor end-plate, with a measure of the density of specific molecules on a membrane (Salpeter 1967).

What is an electron microscope section?

E.m. autoradiography evolved from attempts to apply emulsion layers to conventional e.m. sections. Inevitably, the question arises 'How much of the original tissue survives in the final section?'

The data which might permit one to answer this question are disappointingly scanty. There are hints in the literature that particular proteins—usually enzymes—are removed from tissues by specified fixatives and embedding routines, and that some fixatives are more effective than others at retaining ribonucleic acid. For the most part, however, histologists have accepted the ultra-thin section without questioning too closely the relation it bears, in biochemical terms, to the living tissue. Only in the case of lipids has much work been done, presumably because the solvents used in dehydration and embedding have such a high probability of extracting lipids. Experiments on these lines by a number of laboratories have been summarized by Williams

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(1969). It is clear that variations in the detailed procedure of fixation and embedding can make large differences in the percentage of phospholipids retained in the section. The possibility may even emerge of processing the same labelled tissue in several parallel ways, selectively retaining known percentages of various lipids in each case, in a kind of extractive cytochemistry.

Peters & Ashley (1967) have drawn attention to a possible source of error in experiments on the incorporation of labelled amino acids into protein. The use of glutaraldehyde as a fixative may result in the binding of free amino acids, and perhaps other small molecules also, to structural elements in the tissue. While this is unlikely to be a serious source of error in many experiments, it could lead to significant overestimates of the levels of incorporation in studies with short-term survival. It illustrates one other aspect of the inquiry into the precise composition of the e.m. section, which we so often accept uncritically as a starting-point for autoradiographic experiments.

Cryostat sections for electron microscope autoradiography

The use of fixed and embedded material for e.m. autoradiography limits the range of application of the method. If only we had a technique for cutting fresh frozen material, it might become possible to examine the distribution of drugs, hormones, or even ions at the ultrastructural level. Such methods have been available for some years at the light microscope level (Appleton 1964; Stumpf & Roth 1965), and are giving a new dimension to the type of project that can be undertaken.

Attempts are being made in several laboratories to adapt ultramicrotomes to the task of cutting frozen sections, and preliminary results have been reported by several authors (Appleton 1969; Christensen 1969; Hodson & Marshall 1969). The technical difficulties here are immense. The passage of a cutting surface through a frozen block raises the temperature in a thin zone around the edge by an amount estimated at 100 °C (Hodson & Marshall 1969). Thawing and refreezing in this zone do not have a very serious effect on the distribution of diffusible material in a section 3 or 4 μm thick, as the thawed zone is only a tiny fraction of the section. But with sections in the range of 100 nm or less, this zone extends through most of the section. Cutting will probably have to be carried out at the temperature of liquid nitrogen. Just as important is the control of temperature fluctuations, since these could cause significant variations in section thickness; and in this range variations in thickness will produce proportional changes in grain density.

Claims have already been made that suitably thin sections have been cut from frozen material. This, if true, is only the start. The handling of such delicate sections without thawing, through the stages of emulsion application and exposure, and their subsequent processing and staining, provide formidable difficulties.

The section will freeze-dry, either before or during the early stages of exposure. There may well be deposition of solutes on surrounding membrane systems during this process. Bearing in mind the problems already outlined in analysing the grain distributions in autoradiographs of fixed and embedded material, the difficulties raised by possible movement of the labelled molecule within the tissue could limit the usefulness of the technique.

Work is in progress on developing frozen section techniques for autoradiography, however, and the next few years may see sufficient progress to permit the ultrastructural localization of selected diffusible substances.

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For the present, however, we are restricted in the use of e.m. autoradiography to fixed and dehydrated tissues that have been embedded and sectioned in the conventional way.

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FIGURE 1. (*a*) E.m. autoradiograph of a neuromuscular junction after *in vitro* treatment with [^{3}H]DFP. Ilford L4 emulsion with Microdol X development. Note the large size of the developed grain. (*b*) the same micrograph with hypothetical position of parent crystal and β track. Autoradiograph prepared by Dr M. M. Salpeter.