Short Title:

METHODS FOR QUANTITATIVE E.M. RADIOAUTOGRAPHY

AN ASSESSMENT OF METHODS USED FOR QUANTITATIVE ELECTRON MICROSCOPE RADIOAUTOGRAPHY

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Resolution in electron microscope radioautography is a measure of the accuracy that can be achieved in locating a radioactive source in experimental specimens. The order of resolving power (50 nanometers) being considerably lower than that of the electron microscope itself (0.2-0.3 nm), statistical methods combined with probabilities governed by source-detector geometry, have been applied to the interpretation of silver grain distributions observed in radioautographs. This approach has made it possible to determine an estimate of the location and relative amounts of radioactive label in biological tissues.

In radioautographs, silver grains can be observed to be distinctly related to particular structures (*exclusive* grains), or can be seen more or less overlapping two or more structures (*shared* grains). If all the grains were exclusive only, there would be no need for a statistical analysis to determine the true source of radiation in the tissue. In the case of shared grains, assuming that only one radioactive disintegration is responsible for one grain, then some method must be applied to distinguish which are the labelled structures that were actually responsible for these grains.

The quantitative techniques of Nadler (1971) and Blackett & Parry (1973) are the two main methods designed to evaluate the location and relative content of label in organelles. Both methods require the use of a *resolution boundary circle*, which, when circumscribed around a silver grain is intended to enclose, with some degree of probability, those structures which might have been responsible for the formation of that grain. The size of this circle may be derived experimentally and theoretically. The larger the circle, the greater is the probability of enclosing the radioactive source; but, on the other hand, there will also be an increased number of shared grains making the analysis more complex. Because the selection of the precise diameter of the resolution boundary circle which can be used in either quantitative technique is arbitrary, the influence of the size of the resolution boundary on the accuracy of the analyses was studied by employing a comparatively large (230 nm radius) and small (100 nm radius) circle, as well as a nil circle of 0 nm radius. In the latter case, grains are never shared, so that no further analysis is required. The investigation

was carried out using experimental tissue sections of rat thyroid follicles (kindly provided by Dr. S. Cassol) labelled with ^{55}Fe. An examination was also made of various hypothetical cell models in which a uniform incorporation of isotope was assumed.

Results derived by the quantitative techniques of Nadler and of Blackett & Parry were found to be equivalent despite their different protocols. Moreover, in the majority of cases, the sizes of the resolution boundary circles did not have a significant effect ($p \leq 0.05$) on the analyses. In fact, under certain conditions, the procedure of direct scoring (that is, using no resolution boundary circle and no analysis) provided results which were not significantly different from those achieved after either method of analysis. The interpretation is that when shared grains make up less than 30% of the total number of developed grains for a circle of 100 nm radius, or 50% for a circle of 230 nm radius, then the simple practice of direct scoring of grains is expected to determine as accurate a distribution of the location and relative amount of radioactive label as can be derived after more complex quantitative analyses.

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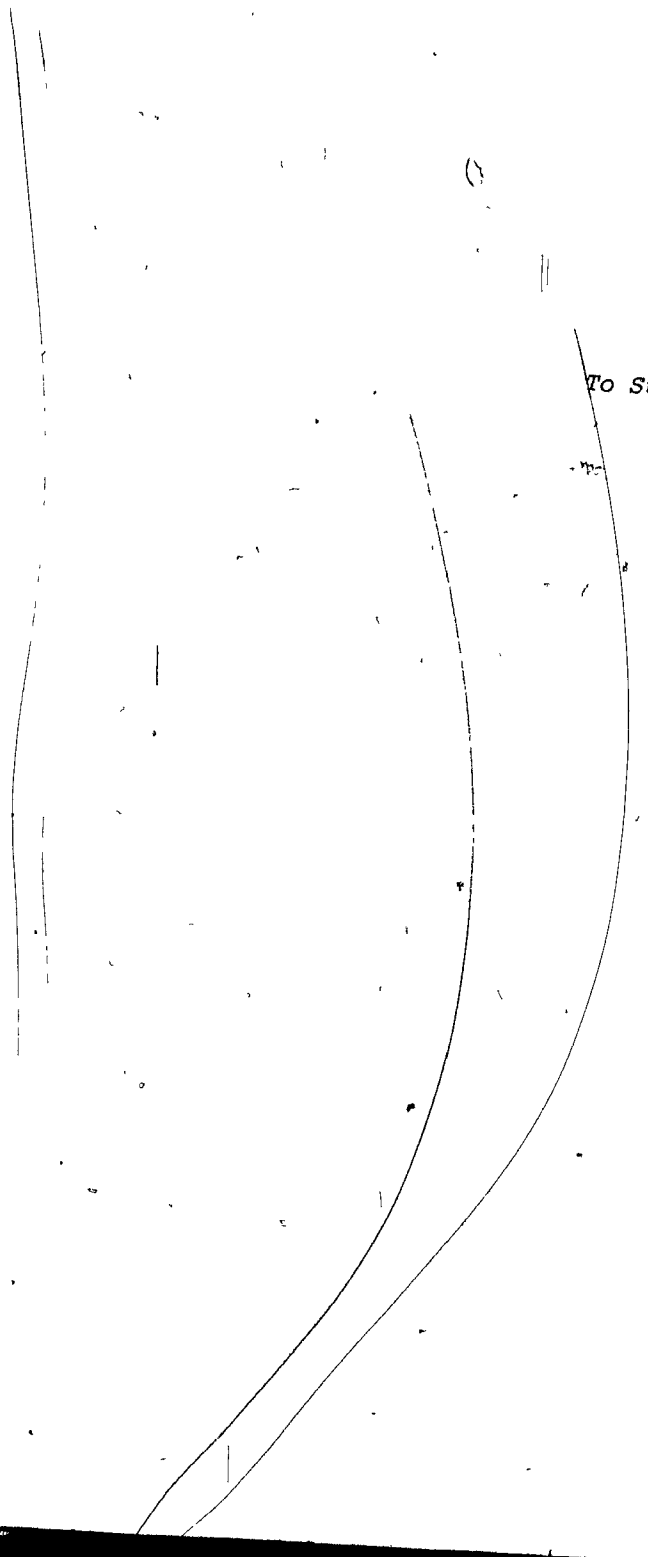
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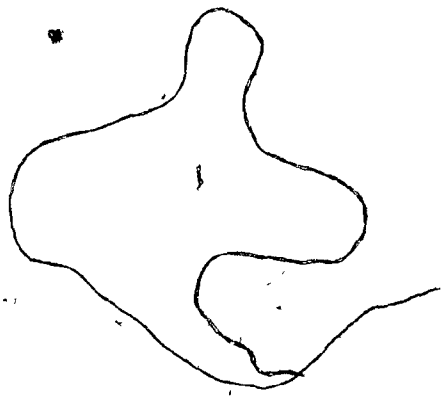
PART III



AN ASSESSMENT OF METHODS USED FOR QUANTITATIVE ELECTRON MICROSCOPE RADIOAUTOGRAPHY

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GENERAL INTRODUCTION

Resolution in electron microscope radioautography is a measure of the accuracy attainable in determining the location of a source of radioactivity within a tissue section. Physical improvements in resolving power have been made through reduction in tissue section thickness, the number of emulsion layers applied to sections, the size of the silver bromide crystal in the emulsion, and the size of the developed silver grain in the radioautograph.

However, even with these improvements, the resolution of electron microscope radioautography is lower than that of the resolution of the electron microscope itself. While resolution of the E.M. is in the order of 0.2-0.3 nanometers (nm), resolution of E.M. radioautography is at best about 50 nm (Salpeter *et al.*, 1977).

Since it is a reasonable assumption that one silver grain is produced by one radioactive emission (Nadler, 1951, 1953; Pelc, 1963; Granboulan, 1965; Bachmann & Salpeter, 1965; Fertuck & Salpeter, 1974), then radioautography is a quantifiable technique. That is, the number of silver grains in a radioautograph is proportional to the amount of radioactivity within a tissue. Furthermore, the number of silver grains produced is also dependent upon the duration of exposure of the photographic emulsion to the radioactive source (Salpeter & Bachmann, 1964; Kopriwa, 1975). Therefore, if the radioautographic grains over cellular structures are counted, it should be possible to correlate the number of grains to the relative proportions of radioactivity contained within those structures.

Due to the random direction of energy particles released by radioactive disintegration from within a tissue treated with a radioactive

tracer molecule, the formation of a silver grain may not occur in the silver bromide crystal directly overlying a suspected source of radiation. It has been shown that grains can be developed at distances of greater than 2 µm from known radiation sources (Caro, 1962; Bachmann & Salpeter, 1964; Granboulan, 1965; Salpeter *et al.*, 1969; Whur *et al.*, 1969; Williams, 1969; Uchida & Mizuhira, 1970; Nadler, 1971), and that assumptions cannot be made about the actual source of radioactivity by virtue of the appearance of a silver grain alone. In addition, the size of the developed silver grain can be larger than the organelle over which it is located, or can overlap two or more organelles, especially in the case of the filamentous silver grain, as opposed to the fine grain.

The filamentous silver grain is produced by using a process of direct chemical development of the radioautograph (James, 1962; Mees & James, 1966). This causes a reduction to elemental silver of the silver bromide in the emulsion and precipitation of the silver, starting at the site of the latent image (Granboulan, 1965), and continuing in the form of a growing filament, which can extend beyond the limits of the silver bromide crystal in which the latent image was situated. Kodak D19b (Eastman Kodak Corp., Rochester, New York) is one of the chemical developers comercially available (Hornsby, 1958).

The fine silver grain is produced by using a solution physical process of development (James, 1962; Mees & James, 1966; Kopriwa, 1975). The developer causes a deposition of silver locally at the site of the latent image and will precipitate silver only within the limits of the individual crystal in which the latent image was formed. Occasionally, several punctate silver deposits can form within the same silver bromide

crystal (Kopriwa, 1975). Agfa-Gevaert solution physical developer (GSPD; Agfa-Gevaert data sheet, Antwerp, Belgium) is one of the fine grain developers manufactured.

Nevertheless, if all the grains in a radioautograph could be assigned exclusively to a single structure (exclusive grains), instead of being shared over more than one structure (shared grains), then grains could be counted and assigned to the structure situated directly beneath the silver grain. However, since this is not the case in practice, there remains the problem of ascribing the grain to its true source of radioactive label within the tissue. Towards this end, methods of quantitative electron microscope radioautography have been developed to interpret silver grain distributions in radioautographs. A quantitative method in electron microscope radioautography is a technique applied to the procedure of grain counting, the goal of which is to determine the location and amount of radioactivity within a tissue as precisely as possible. With this tool, it has been made possible to calculate the most likely sources and relative quantities of radioactivity in a specimen, by application of probability factors and statistical analyses to grain counting methods, which establish the distribution of grains in radioautographs.

The subject of this thesis is an investigation into the quantitative approach to electron microscope radioautography. Studies were carried out on the methods in current use for the quantitative analysis of electron microscope radioautographs in order to establish the relative merits of these techniques. The text of this investigation has been divided into three parts.

Part I deals with the preparation and application of artificial *line source* calibration specimens of radioactive isotopes (Salpeter *et al.*, 1969). These are homogeneously labelled profile sections in plastic, which are coated with photographic emulsion, exposed and developed as radioautographs. Probability curves are determined from the pattern of grain distribution around the known source of radioactivity. Distances of grains from the known source of radiation can be ascertained within which certain proportions of the total number of developed grains are found. The percentage of grains can be correlated to their distance away from the line source. These data can then be used as a standard test specimen which can be compared to unknown sources of radiation in experimental tissues.

Values for *HD* or "half-distance" (the distance from the line source within which 50% of all developed grains are found; Salpeter *et al.*, 1969) were determined under various experimental conditions. The problems of interpretation and measurement of silver grains at distances from the line source, inherent in this form of analytical technique, were investigated. In addition, the general applicability of the method to electron microscope radioautography was examined.

Parts II and III involve an analysis of the methodology and application of two of the available quantitative techniques, specifically, the method of Nadler (1971) and the method of Blackett & Parry (1973).

In Part II, samples have been studied from experimental tissues. Thyroid glands of male Sherman rats, four hours after injection of $^{55}FeCl_3$, were employed as a 'substrate' for testing and comparing both quantitative techniques. (E.M. radioautographs kindly supplied by Dr. S. Cassol.)

Part III is based on the analysis of specific hypothetical cell models, or hypothetical tissue standards, in the form of composite photographs constructed from biological tissue samples after the hypothetical introduction of a radioactive tracer substance (see Materials and Methods).

It was decided to use such hypothetical cell models as a test system for comparing the quantitative methods since, in this way, it was possible to maintain rigid conditions which could be manipulated at will, and, more importantly, which would simplify actual grain counting by eliminating the following:

Uncertainty in *differentiating structures* underlying a silver grain.

Uncertainty of *identification* where silver grains completely obscure underlying structures.

Abundance of different organelles.

Variations in size and shape of different organelles.

Varying distribution ("diffusivity")of organelles.

Variations in section thickness.

Variations in emulsion thickness and uniformity.

Variations in exposure or development duration.

Any effects of background radiation or scatter.

Effects of differences in isotope.

By using hypothetical tissue standards, the testing system can be simplified, idealized and none of the above-mentioned routine problems are encountered, since:

Organelles are chosen in advance and known without doubt.

Hypothetical grains cannot obstruct underlying structures.

Quantity of organelles can be limited arbitrarily.

Standardized, recorded variations in size and shape of organelles are reproduced photographically for comparative analysis.

Diffusivity of organelles is randomized with the aid of a computer.

There are no tissue sections.

There is no emulsion.

No exposure or development is necessary.

No radioactivity is actually used, therefore there can be no background or scatter.

Since isotopes are theoretical, incorporation of label is based on the assumption of uniform uptake of label (Blackett & Parry, 1973) and comparisons can be made with this rationale.

Under both experimental and hypothetical conditions, the concept of the resolution boundary circle, also known as the probability circle (Caro, 1962; Caro & Schnös, 1965; Granboulan, 1965; Salpeter *et al.*, 1969; Whur *et al.*, 1969; Williams, 1969; Nadler, 1971; Blackett & Parry, 1973) was studied in order to determine its relevance in quantitatively assessing radioautographic data. The resolution boundary circle is a circle of certain radius, such that when superimposed over a silver grain in an E.M. radioautograph, it will contain within its bounds those structures having possibly been the source of radioactivity which caused that grain to form. The size of the circle is dependent upon an arbitrarily chosen probability which may be derived on theoretical bases, or by experimental observation. The size of the circle may vary according to the type of isotope, emulsion and developer used in a particular study as well.

INTRODUCTION - PART I

i) *History*

In 1969, Salpeter *et al.* introduced a procedure for the preparation of a calibration specimen for testing resolution in electron microscope radioautography. A radioactive polystyrene film, approximately 50 nm thick, was used as the known source of radiation. Embedded between methacrylate and Epon, the 'sandwich' specimen was sectioned at right angles, resulting in what was termed a *line source*, or "hot line" of radioactivity. In the electron microscope, the line could be seen clearly without staining. The sections were coated with photographic emulsion, exposed and developed. The developed radioautographs were photographed in the electron microscope along the full length of the hot line, which yielded over 1,000 developed grains. The distance from the mid-point of every grain to the middle of the line source was measured up to a "cut-off" distance (Salpeter *et al.*, 1969) of 2 µm on either side of the line. The distance from the line within which half the number of developed grains were observed was called the half-distance, or *HD*. The grains were then recounted, this time incorporating a "cut-off" distance of 10 HD units. This final HD value derived was then used as a direct measure of resolution. Universal curves were plotted graphically to demonstrate the distribution of developed silver grains around the line source. The universal curves could then be compared to silver grain distributions in experimental radioautographs (Budd & Salpeter, 1969) to demonstrate non-random incorporation of radioactive label within certain cellular compartments.

ii) *Problems to be Investigated*

Line sources were prepared with modifications to the Salpeter technique. Several isotopes, emulsions and developers, used most often in this

laboratory (Department of Anatomy, McGill University, Montreal, Canada) were studied and their effects on resolution evaluated. The determination of specific HD values was performed under these various conditions.

Throughout the study, the line source method was analysed, taking into account possible problems, as well as those problems actually encountered when interpreting silver grain distributions. The assessment of the centre of the developed silver grains and the location of the mid-point of the thickness of the line source (ca. 50 nm) posed difficulties. In addition, the validity of these points of reference for measurements was investigated. Possible errors in performing measurements and reading them from a metric scale were also examined. The effects of altering the quantity of grains counted, and altering the distance from the line source beyond which grains were excluded from the counts (cut-off distance; Salpeter *et al.*, 1969), were determined.

The practical limitations of the technique of Salpeter regarding resolution in electron microscope radioautography included the necessity of preparing a radioactive line source, measuring the distances of developed silver grains from the line in E.M. radioautographs, repeating the protocol for measuring the distances of grains away from specific organelles in an experimental specimen under study and comparing these latter distributions to that of the expected. Inherent in making these measurements was the possibility of introducing errors during the interpretation of silver grain distributions.

More importantly, however, the technique offered no information about the relative content or concentration of radioactive label with

respect to various organelles within a tissue, but allowed only the designation of a non-random pattern of the distribution of silver grains around an organelle suspected as the radioactive source.

In summary, an investigation of the methodology of line source radioautography was carried out, with special attention being paid to the technical and physical problems of interpreting in practice, distributions of silver grains around a known radioactive source.

PART I

MATERIALS AND METHODS

Line Source Evaluation and Determination of HD Values in Electron Microscope Radioautography

A. *Preparation of Radioactive Source*

i) ^{3}H as the Source of Radiation

Blocks of poly-n-butyl methacrylate, labelled with tritium at a concentration of 10 mCi/gm (Amersham/Searle), were used as the source of radiation for tritium studies. Homogeneity of label was assured by the manufacturers, and was confirmed by Dr. B. Kopriwa in unpublished tests (1973).

ii) ^{125}I as the Source of Radiation

The source of activity intended for ^{125}I studies was the radioiodinated colloid of rat thyroid follicles. Female Sherman rats, weighing approximately 150 grams, were given intraperitoneal injections of 10 µCi of ^{125}I per gram of body weight (in sodium iodide solution, at a concentration of 5 mCi/ml; specific activity was 1.5 X 10^{7} µCi/mg; Charles E. Frosst, Montreal, Canada.)

Twenty-four hours after the injection, the rats were anaesthetized with ether, and sacrificed by intra-cardiac perfusion of 2.5% glutaraldehyde (Sabatini *et al.*, 1963) (25% glutaraldehyde, TAAB Laboratories, Reading, England), in 0.2M Sörensen's phosphate buffer, pH 7.2-7.4 (Gomori, 1955), with 0.1% sucrose and 0.25% dextrose (Fisher Scientific Co., Montreal, Canada) added to increase the tonicity of the solution to 440 milliosmoles per Kg. (Chajut, N., 1972). Perfusate circulated by means of a perfusion pump (Ministaltic Pump, Cat. No. 72-894-21, Manostat Corp., New York) for an initial 5 minutes, and continued by gravity for an additional 15 minutes.

The thyroid glands were then removed *en bloc* with the trachea, and immersed for two hours in the same fixative at 4°C. With the aid of a Zeiss dissecting stereo-microscope (Mag. 10X - 40X), the thyroid lobes were removed from the trachea, gently trimmed of connective tissue, and chopped into 1 mm cubes with a razor blade. The tissue was then washed for 4 hours at 4°C, in half-hour changes of 0.15M Sörensen's phosphate buffer washing solution (Gomori, G., 1955) and left in buffer overnight. It was then treated in a 1% solution of osmium tetroxide (Millonig, G., 1962) from a 4% OsO_4 solution (British Drug House, Montreal, Canada) in buffer for 2 hours at 4°C. The tissue was dehydrated in graded solutions of ethanol, from 30% up to 100%, and in two final changes of propylene oxide.

The tissue was infiltrated in a propylene oxide: Epon mixture of 3:1 for 3 hours, then 2:1 for 3 hours, and a 1:1 mixture overnight. Following this, was a 1:2, a 1:3 and a final pure Epon mixture (Luft, J. H., 1961), each infiltrating for 3 hours at room temperature. The tissue was then embedded in evacuated Epon in Beem capsules, and polymerized in a 60° oven for 72 hours.

B. *Preparation of Sections for Line Source Radioautography*

(Acknowledgements to Dr. B. Kopriwa for radioautographic and electron microscope work, and to Mrs. L. Volkov for cutting sections.)

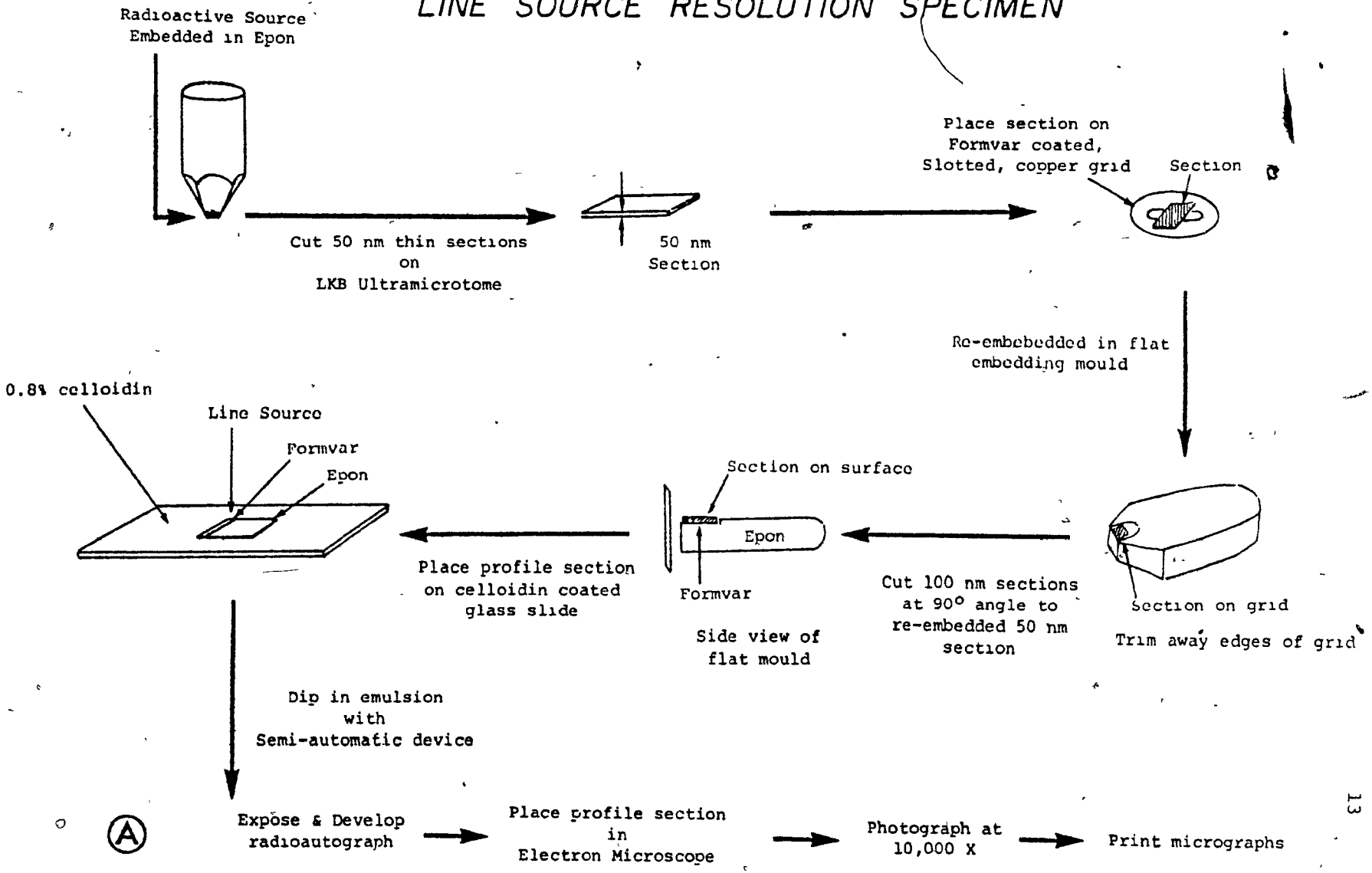
In experiments involving ^{3}H and ^{125}I blocks, *line sources* were prepared with modifications to the method of Salpeter (1969). Ultra-thin (silver) sections of about 50 nm were cut with a diamond knife (Dupont Co., Wilmington, Delaware) on an LKB ultra-microtome (LKB-Produkter, Stockholm, Sweden) and

subsequently re-embedded in Epon, in flat embedding moulds, on Formvar coated, slotted, copper grids. During the process of re-embedding, the radioactive source was embedded at the surface of the Epon, with the Formvár and Epon in contact with the section of its undersurface. These were then resectioned at a 90° angle to the original cut, to produce profile sections of pale gold interference colour, approximately 100 nm thick. These resulting sections were placed on celloidin coated (0.8% celloidin in iso-amylacetate) glass slides, such that the profile of the line source was bordered on one side by Epon, and on the other side only by celloidin. Developed silver grains appearing on either side of the line source were interpreted as being over either the Epon side of the line or over the celloidin side. This arrangement was slightly different than the "hot line sandwich" described by Salpeter *et al.* (1969) (see Fig. I-A).

The celloidin coated slides with the sections on them were then dipped in various emulsions with a semi-automatic device (Kopriwa, B., 1967) expected to produce a uniform monolayer of silver bromide crystals. Specifically, the emulsions used were Ilford L4 (Ilford Ltd., Ilford, Essex, England) and Kodak NTE (Eastman Kodak Corp., Rochester, N. Y.). The radioautographs were exposed for periods from 12 through 103 days to assess optimal grain densities. They were developed with two different types of developers producing either a filamentous grain (Hornsby, K. M., 1958) with Kodak D19b, or a fine grain (James, 1962; Mees and James, 1966) with Agfa-Gevaert solution physical developer ("GSPD"; Agfa-Gevaert Data Sheet, Antwerp, Belgium; modified by Kopriwa, 1975). Fine grain development was always preceded by gold latensification (James, 1948; James *et al.*, 1948; Hamm & Comer, 1953; Salpeter & Bachmann, 1964; Bachmann & Salpeter, 1965)

FLOW DIAGRAM

PROCEDURE FOR PREPARING RADIOACTIVE LINE SOURCE RESOLUTION SPECIMEN



in freshly prepared gold thiocyanate (Salpeter and Bachmann, 1964; Rechenmann and Wittendorp, 1972, 1976; Kopriwa, 1975).

A table outlining the various procedures follows:

Table I-A

Isotope	Emulsion	Developer	Exposure Time (Days)
^{3}H	L4	GSPD	49
		D19b	25, 49, 63
	NTE	D19b	103
^{125}I	L4	GSPD	12, 20
		D19b	18

Unstained sections were placed in a Hitachi HU-11C electron microscope, and photographs were taken at a magnification of 10,000X along the length of the 50 nm thick line source, then processed and developed to give a final magnification of 25,000X on 8" X 10" prints on Ilford photosensitive paper.

For each study, approximately 70 to 100 photographs were produced including from 4 to 30 grains per photograph. The intention was such that the total grain counts would approach a value of about 500.

C. Quantitation and Measurement

The following method for measuring the grain distribution around the line source was implemented. A Zeiss dissecting stereo-microscope was used in order to further increase the magnification of the micrographs.

In all photographs the centre of the line source was determined as

closely as possible across its thickness, and a fine scratch mark was made along its length with the point of a 25 gauge syringe needle (Becton, Dickinson and Co. Canada Ltd., Mississauga, Canada).

The next task was to locate the centre of the developed silver deposits. In the case of the filamentous grain, a transparency of concentric circles, placed over the grain, indicated the geometric centre of the developed filament. With the solution physical developer, fine grains formed either singly or in small clusters, usually composed of two or three silver deposits (Kopriwa, 1975). The centre of a single silver deposit was assigned without difficulty. In order to ascertain the centre of a cluster of silver deposits, a transparency of a circle with a diameter equivalent to the average size of the silver bromide crystal in the emulsion was superimposed onto the photograph. For Ilford L4, the average crystal size was 0.14 μm (Kopriwa, 1975). If the whole cluster of silver deposits fit within the bounds of the transparency, then the centre of the circle (crystal) was chosen as the point of measurement from the line. If the whole cluster did not fit completely within the confines of the circle, then some of the silver deposits were assigned as those developed from an adjacent crystal. The centre of these were then determined in like manner.

Using a metric measuring magnifier (Bausch & Lomb, U.S.A., Cat. No. 81-34-35, 81-34-38), distances were measured from the centre of the grain to the centre of the line source to the nearest 0.05 mm. Accounting for the magnification of the photographs, 1 mm was equivalent to 40 nm in distance, and thus possible error, due to reading of the actual measurements was limited to within 2 nm. Grain distances were measured from the centre of the line on either side, but since their distributions were

found to be similar (see Results), they were accumulated to determine one-sided displacements. All grains were numbered and their distance from the line recorded. In this way, reproducibility of the system of measurement was verified.

Results were tabulated in the form of grain densities per unit distance from the line source, and histograms, graphs and other tables were derived from this information.

Where applicable, statistical comparisons of the data were made using a χ^2 test for two sets of observed values (Freund, J. E., 1962), as shown in the following formula:

$$\chi^2 = \frac{N}{N_A}\left[\frac{a_1^2}{N_1} + \frac{a_2^2}{N_2} + \cdots + \frac{a_k^2}{N_k}\right] + \frac{N}{N_B}\left[\frac{b_1^2}{N_1} + \frac{b_2^2}{N_2} + \cdots \frac{b_k^2}{N_k}\right] - N$$

where N_A = Total of observations in Group A

N_B = Total of observations in Group B

N = Total of observations in A and B

$a_1, a_2 \ldots a_k$ = Individual observations in A

$b_1, b_2 \ldots b_k$ = Individual observations in B

degrees of freedom (df) = k-1

This formula was applied to an electronic calculator program for the Hewlett-Packard Calculator, Model 10, with the 9810A StatPac (Hewlett-Packard Co., Calculator Products Division, Loveland, Colorado), and was used to carry out the necessary analyses.

RESULTS - PART I

Results of the various procedures are shown in Tables I-1 to I-5. Histograms and universal curves illustrating the data are shown in Figures I-7 to I-16.

i) Effects of Isotope on Resolution

In these studies, ^{125}I demonstrated a better resolution than did ^{3}H, only when using the Kodak D19b developer which produced a filamentous type of grain. The HD value for ^{125}I was 157 nm, while the HD for ^{3}H was 187 nm.

When using the GSPD developer, which produced a punctate, fine type of grain, the HD values for ^{125}I and ^{3}H were not considered to be different, with ^{3}H having an HD of 76 nm, and ^{125}I having an HD of 80 nm.

ii) Effects of Emulsion on Resolution

One preliminary study was carried out with respect to the effects of emulsion on resolution. The Kodak NTE, an emulsion characterized by a relatively low sensitivity, composed of small diameter (0.05 μm) silver bromide crystals, resulted in an improvement in resolution to an HD value of 119 nm over that of the Ilford L4 emulsion (crystal diameter of 0.14 μm), with an HD of 187 nm.

iii) Effects of Developer on Resolution

Of the two kinds of commercially available developers investigated, Kodak D19b and Agfa-Gevaert solution physical developer (GSPD), the GSPD demonstrated HD values which were about half the values of those produced with the D19b. This improvement was consistent for both isotopes studied. For ^{3}H, where the D19b resulted in an HD value of 187 nm, the GSPD gave an HD of 76 nm. For ^{125}I, where D19b produced an HD value of 157 nm, the GSPD showed an HD of 80 nm.

iv) Effects of Absorption of Radiation by Epon on Resolution

Figures I-12 to I-16 are histograms of the normalized grain density per unit area in relation to distances away from the line source, on both sides of the line, being either the "Epon" side or "celloidin" side. In order to derive normalized distributions, the maximum actual grain count from each study was designated as 100%. All lower counts for unit distances away from the line were expressed as percentages of this value. In this way data could be compared regardless of the actual number of grains within each study or unit distance away from the line source.

The data were analysed by a χ^2 for two sets of observed values ($p = 0.05$). Results from Table I-2 demonstrate that there was no significant difference between the distribution of grains over the Epon side as compared to that over the celloidin side of the line source, in all cases except where the Kodak NTE was used. In this case there was a significant difference in the normalized distribution, but no significant difference between the actual grain counts. Of the 137 grains counted, however, only 63 were over the Epon side, while there were 75 grains over the celloidin side.

v) Effects of Exposure Time on Resolution

A study was made with ^3H, the Ilford L4 emulsion and the Kodak D19b developer, on the effects of exposure time on resolution, as shown in Table I-3. At 25 days exposure, an HD value of 104 nm was considered to be low and may have reflected the low density of grains available for counting. After 49 days exposure, an HD of 193 nm was found. After 63 days exposure, the HD was 190 nm. The difference between 49 and 63 day exposure periods was negligible.

vi) *Effects of Cut-off Distance on Resolution*

Again using the ^{3}H, L4, D19b experiment, a study was made on the effects on HD of altering the cut-off distance for grain measurements. A cut-off distance was arbitrarily chosen at 600 nm, or about 3 HD, as opposed to a distance of 10 HD as suggested by Salpeter *et al.* (1969). A comparison by cross-reference with various exposure times demonstrated that reduction of the cut-off distance by this amount reduced the definitive HD values by about 20 to 30 nm. At 49 days exposure, the HD was reduced from 193 nm to 170 nm by cutting off counts at 3 HD. At 63 days exposure, the HD fell from 190 nm to 160 nm. The pooled results from all exposure times showed a drop in HD from 187 nm to 160 nm, by altering the cut-off distance to 3 HD.

vii) *Effects of Quantity of Grain Counts on Resolution*

The data from the study using ^{3}H with the L4 emulsion and D19b developer was analysed in relation to the effects of altering the quantity of grains included in the measurements. Regarding the minimum number of grains that can be counted and still produce valid results, inspection of Table I-3 indicates that counting only 50 grains considerably diminished the HD value, while counting 257 grains produced essentially the same result as for counting 677 grains.

A cursory look at data from the Kodak Nuclear Track Emulsion in Table I-2, indicates that counting 137 grains may not have been enough, due to the difference between counts over the Epon and celloidin sides of the line.

In the study with ^{125}I and the L4 emulsion, developed with GSPD, a preliminary count including 355 grains with no cut-off distance resulted in a HD of 86 nm. A second count, which included an additional 178 grains,

giving a total of 533, again with no cut-off distance, resulted in an HD of 84 nm. This difference was considered to be negligible and indicated that counting 350 grains was sufficient to produce the same results as derived by counting about 550 grains.

TABLE I-1

Effects of Isotope, Emulsion and Developer on HD

Isotope	Emulsion	Developer	HD (nm)	No. Grains Counted
^{3}H	L4	D19b	187	677
		GSPD	76	439
	NTE	D19b	119	137
^{125}I	L4	D19b	157	286
		GSPD	80	512

TABLE I-2

Results of χ^2 tests on Normalized Grain Distribution Patterns on either side of the Line Source

Isotope	Emulsion	Developer	*χ^2 Significance ($p = 0.05$)
^3H	L4	D19b	n.s.
		GSPD	n.s.
	NTE	D19b	s.
^{125}I	L4	D19b	n.s.
		GSPD	n.s.

*n.s. = not significantly different

s. = significantly different

TABLE I-3

Effects of Exposure Time on Resolution

Isotope, Emulsion & Developer	Exposure Time (Days)	Number of Grains	HD (nm)
^{3}H, L4, D19b	25	50	104
	49	257	193
	63	370	190
	all times, pooled data	677	187

TABLE I-4

Effects of Cut Off Distance on Resolution

Isotope, Emulsion & Developer	Exposure Time (Days)	Number of Grains	HD if cut off at (nm) 3 HD
^{3}H, L4, D19b	25	46	88
	49	226	170
	63	322	160
	all times, pooled data	594	160

Figure Legends - Part I

Figure I-1. Profile section of ^{3}H labelled methacrylate, 50 nm thick, acting as a *line source*. (Kindly supplied by Dr. B. Kopriwa).

The radioautograph was dipped in Ilford L4 emulsion, and developed with Kodak D19b, (X 25,000).

LS = line source

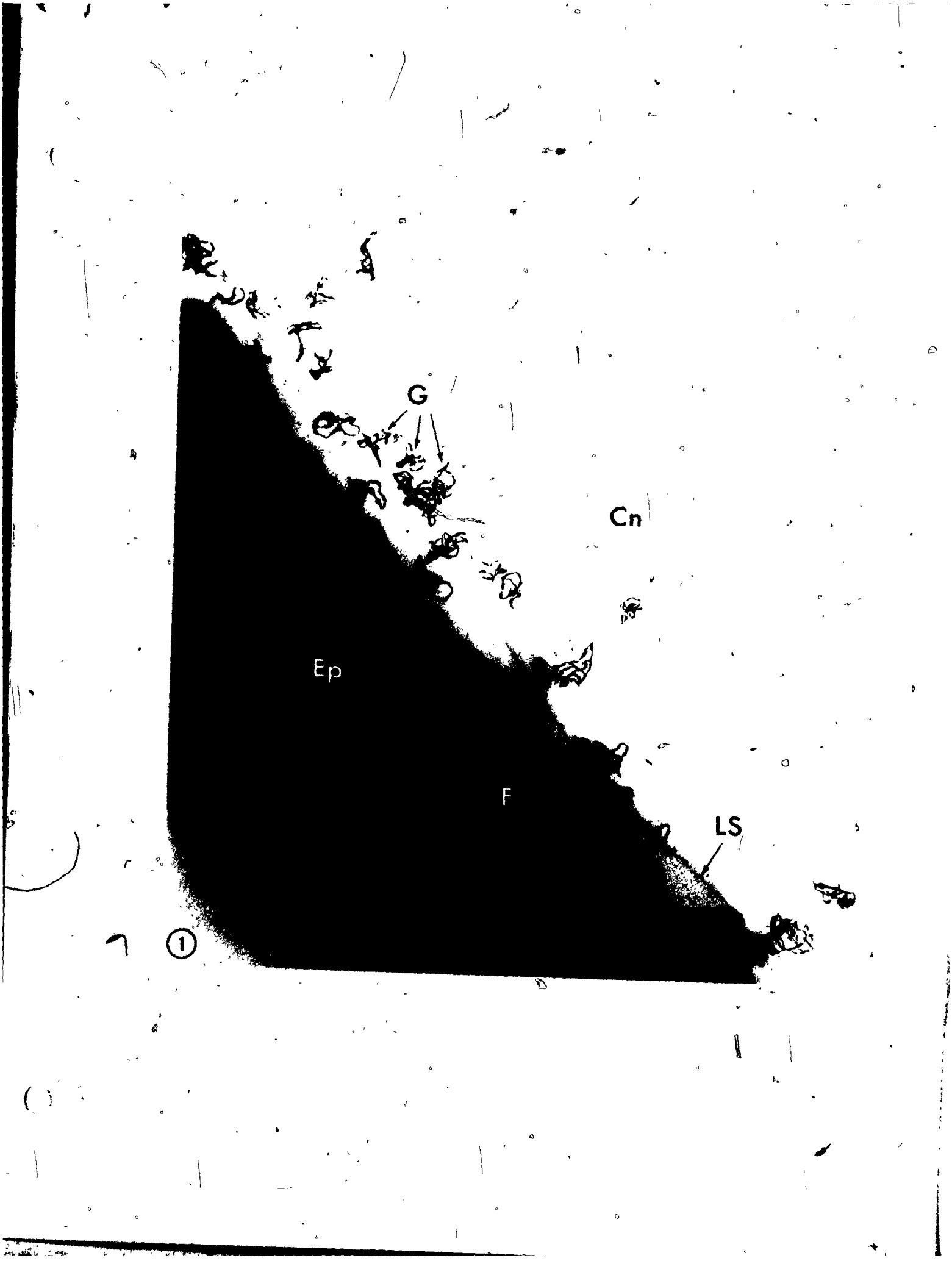
Ep = Epon side of line source

Cn = Celloidin side of line source

F = Formvar

G = Filamentous grains

Distribution of grains results in an HD value of 187 nm. There was no significant difference between distributions of grains over the Epon and celloidin sides of the line.


G
Cn
Ep
F
LS
1

Figures I-2 to 6. Various preparations of *line sources* used in these studies. (X 30,000)
Line source indicated by double arrows.

Figure I-2. Line source of ^{3}H labelled methacrylate, dipped in Ilford L4 emulsion and developed with Kodak D19b. As seen in previous figure, the grain distribution provided an HD value of 187 nm.

Figure I-3. Line source of ^{3}H labelled methacrylate, dipped in Ilford L4 emulsion and developed with GSPD. An HD of 76 nm indicated an improvement in resolution over the chemical developer. Note clusters of two or more silver deposits derived from individual silver bromide crystals.

Figure I-4. Line source of ^{125}I labelled thyroid follicular colloid (rat), dipped in Ilford L4 emulsion and developed with Kodak D19b. An HD of 157 nm indicated a better resolution than found with ^{3}H.

Figure I-5. Line source of ^{125}I labelled thyroid follicular colloid, dipped in Ilford L4 emulsion and developed with GSPD. Resolution was not considered to be different than for ^{3}H under the same conditions and was better than that found with the chemical developer. An HD of 80 nm was determined.

Figure I-6. Line source of ^{3}H labelled methacrylate, dipped in Kodak NTE emulsion and developed with D19b. Small diameter silver bromide crystals (0.05 μm) gave rise to small filamentous developed grains. The HD was found to be 119 nm.

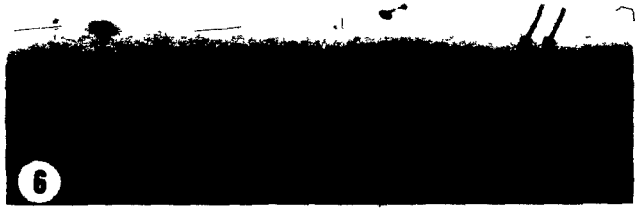
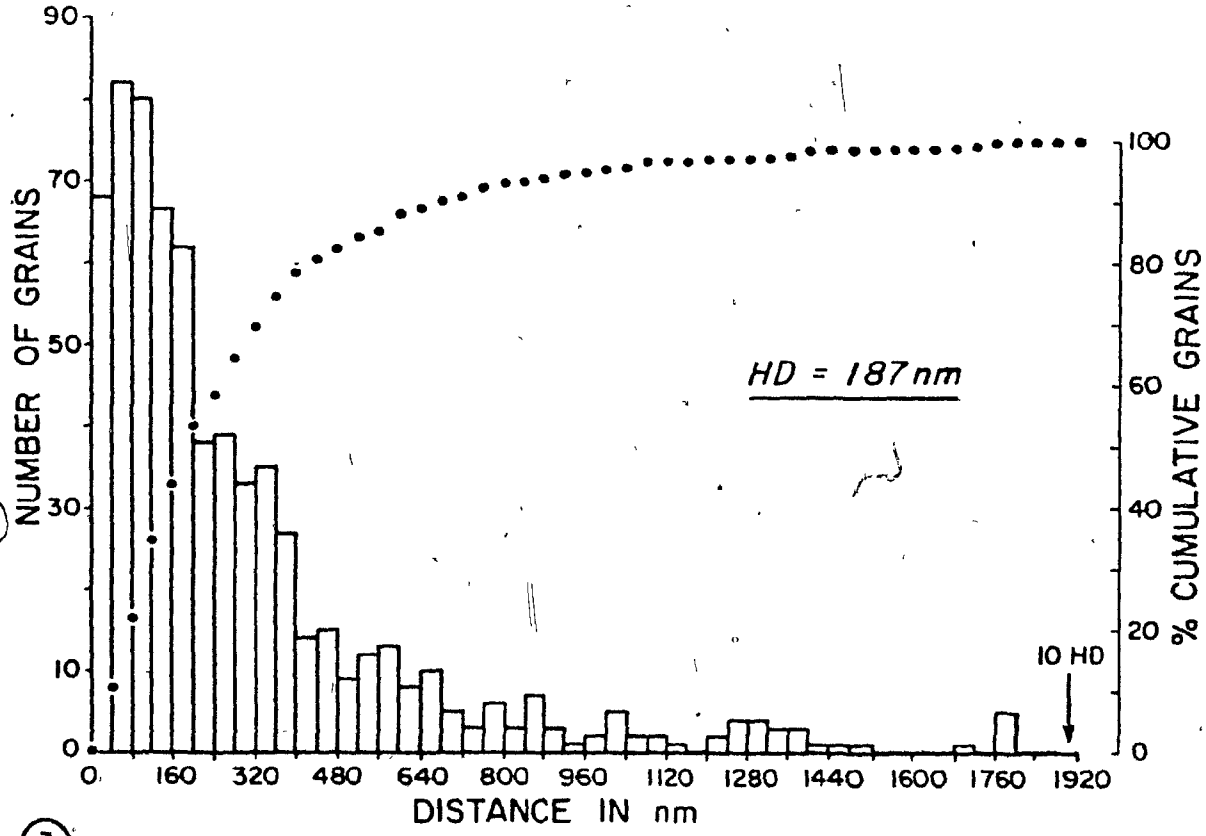


FIGURE LEGENDS - PART I

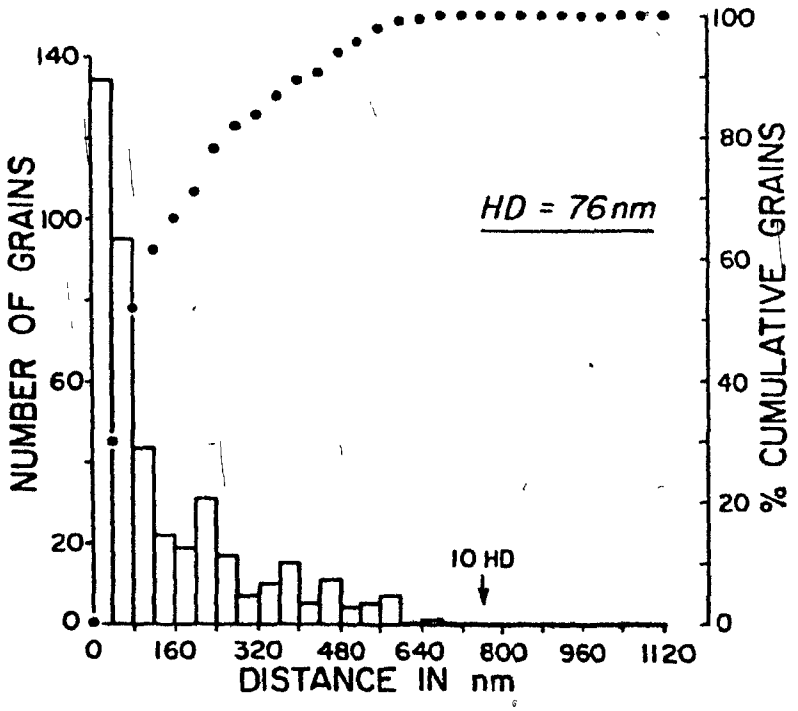
Figures I-7 - I-11 These graphs show the experimental density distributions of silver grains around various radioactive *line sources* used in these studies. The left-hand ordinate corresponds to the histogram presenting the actual number of grains observed per unit distance from the line. The right-hand ordinate corresponds to the integrated grain distribution showing the cumulative percentage of grains in relation to distance. A cut-off distance of 10 HD was used throughout.

Figure I-7 This histogram shows the grain density distribution for the isotope ^{3}H, where the line source specimen was dipped in Ilford L4 emulsion and the radioautograph was developed in Kodak D19b, producing a filamentous grain. Note the long "tail" of the curve. An HD of 187 nm acts as a measure of resolution.

Figure I-8 This graph shows the grain density distribution for a ^{3}H line source, again dipped in Ilford L4 but developed with GSPD, giving rise to a fine grain. An HD of 76 nm indicates an improved resolution over the D19b developer.


NUMBER OF GRAINS
% CUMULATIVE GRAINS
HD = 187nm
10 HD
DISTANCE IN nm
0
10
30
50
70
90
0
20
40
60
80
100
0
160
320
480
640
800
960
1120
1280
1440
1600
1760
1920

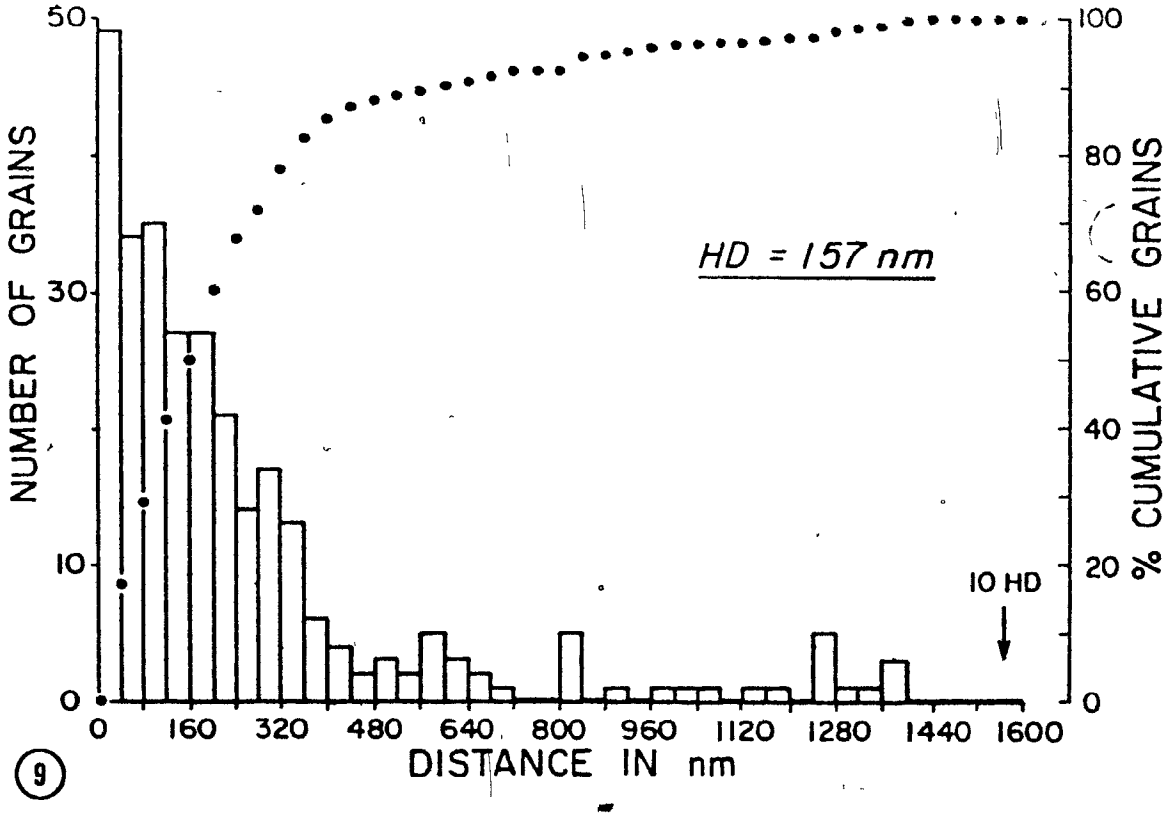
(7)


NUMBER OF GRAINS
% CUMULATIVE GRAINS
HD = 76nm
10 HD
DISTANCE IN nm
0
20
60
100
140
0
20
40
60
80
100
0
160
320
480
640
800
960
1120

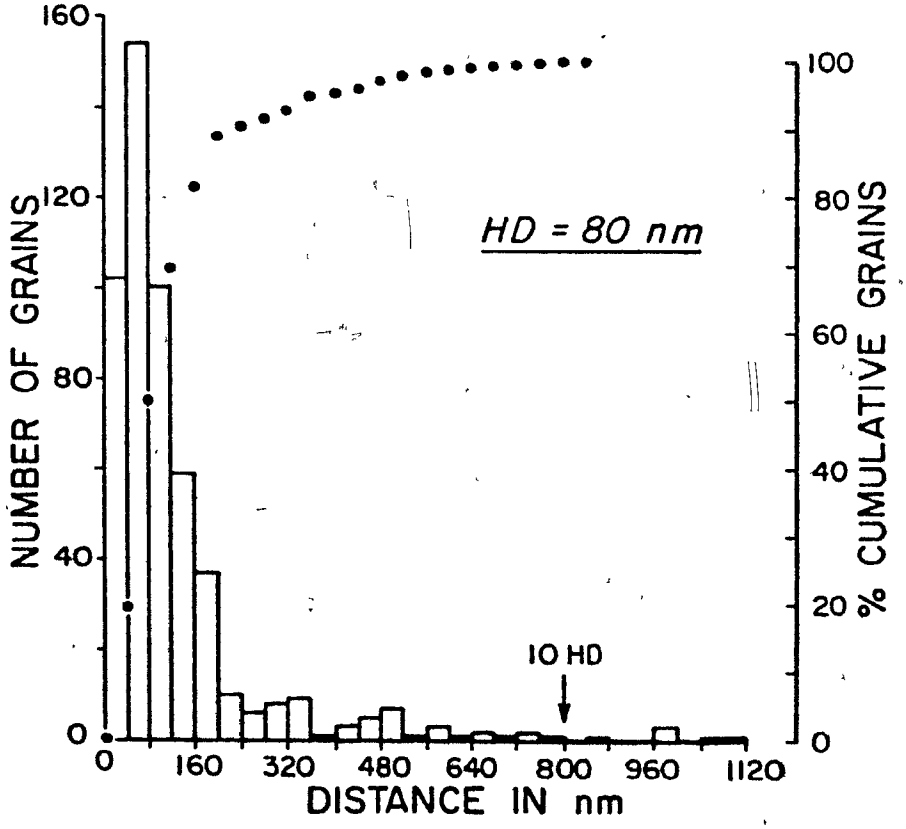
(8)

Figure I-9 This graph shows the pattern of distribution for grains developed by D19b after exposure of an ^{125}I line source to Ilford L4 emulsion. The observed HD of 157 nm indicates that better resolution is achieved with ^{125}I over ^{3}H, under similar conditions.

Figure I-10 The histogram and integrated percentage of cumulative grains demonstrates the distribution for an ^{125}I line source dipped in Ilford L4, and developed by the Agfa-Gevaert solution physical method. An HD of 80 nm was determined from the pattern of these fine type of grains. This implies that with GSPD there are no significant differences in resolution caused by the isotopes ^{125}I and ^{3}H. The resolving power obtained with GSPD is about two-fold better than when developed by D19b.

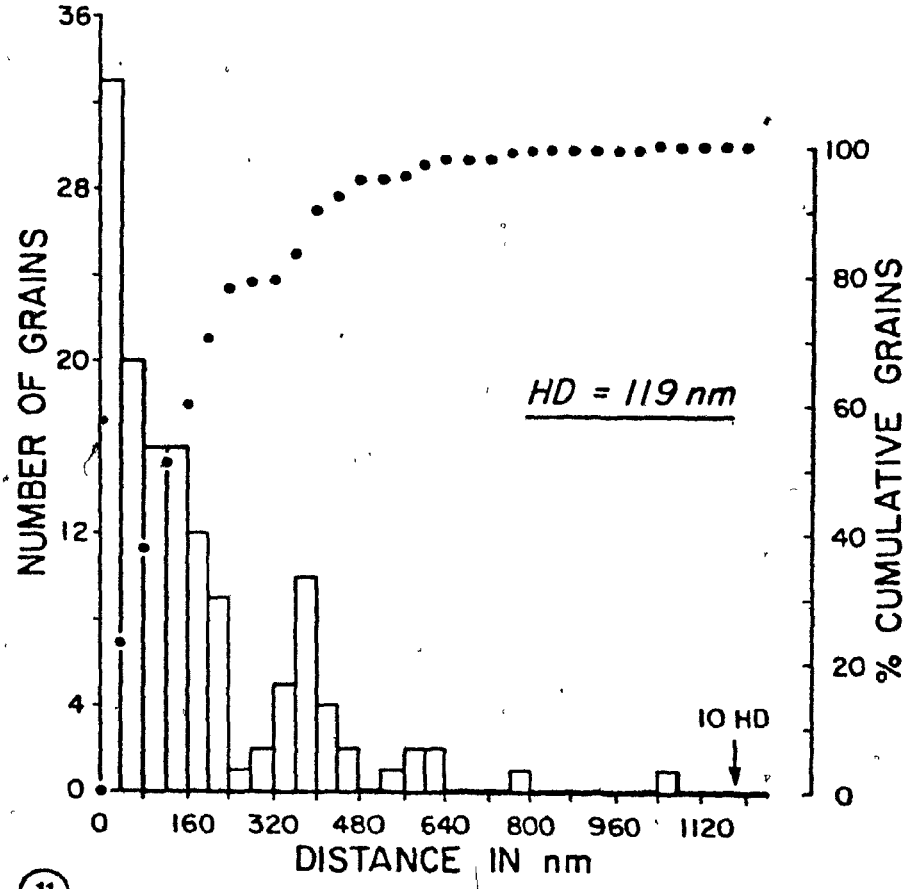

NUMBER OF GRAINS
% CUMULATIVE GRAINS
HD = 157 nm
10 HD
DISTANCE IN nm
0
10
30
50
0
20
40
60
80
100
0
160
320
480
640
800
960
1120
1280
1440
1600

(9)


NUMBER OF GRAINS
% CUMULATIVE GRAINS
HD = 80 nm
10 HD
DISTANCE IN nm
0
40
80
120
160
0
20
40
60
80
100
0
160
320
480
640
800
960
1120

(10)

Figure I-11 This graph demonstrates the distribution of grains for a ^{3}H line source dipped in Kodak NTE emulsion and developed by the direct chemical procedure with D19b. This produced a comparatively small filamentous type of grain. Resolution with NTE was better than with the Ilford L4 emulsion, as shown by an HD of 119 nm. However, this emulsion provides inconsistent results and its low sensitivity makes it impractical for radioautography.

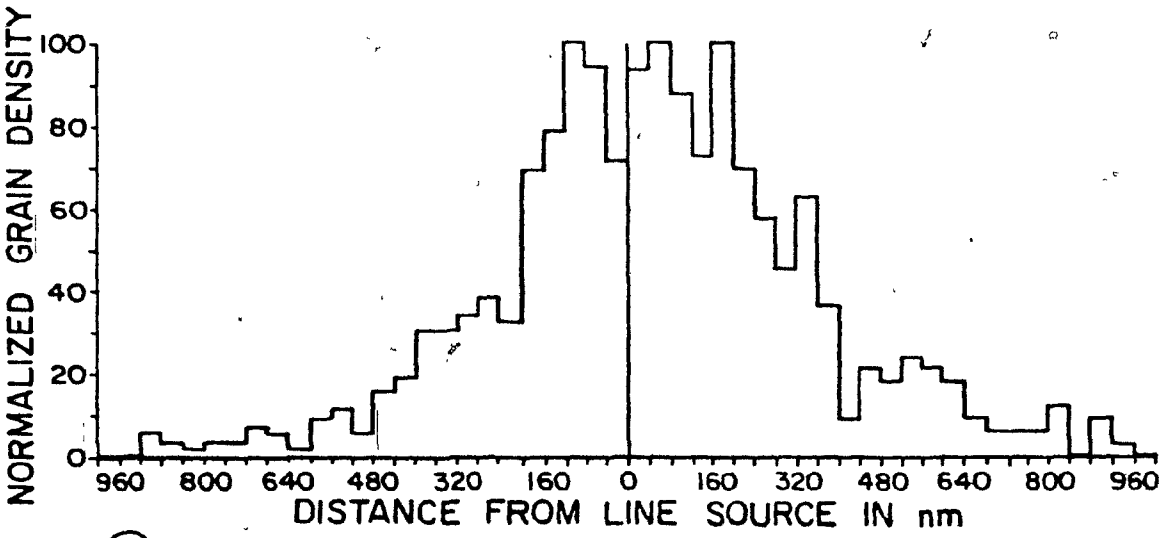

36
28
20
12
4
0
NUMBER OF GRAINS
100
80
60
40
20
0
% CUMULATIVE GRAINS
HD = 119 nm
10 HD
0
160
320
480
640
800
960
1120
DISTANCE IN nm

(11)

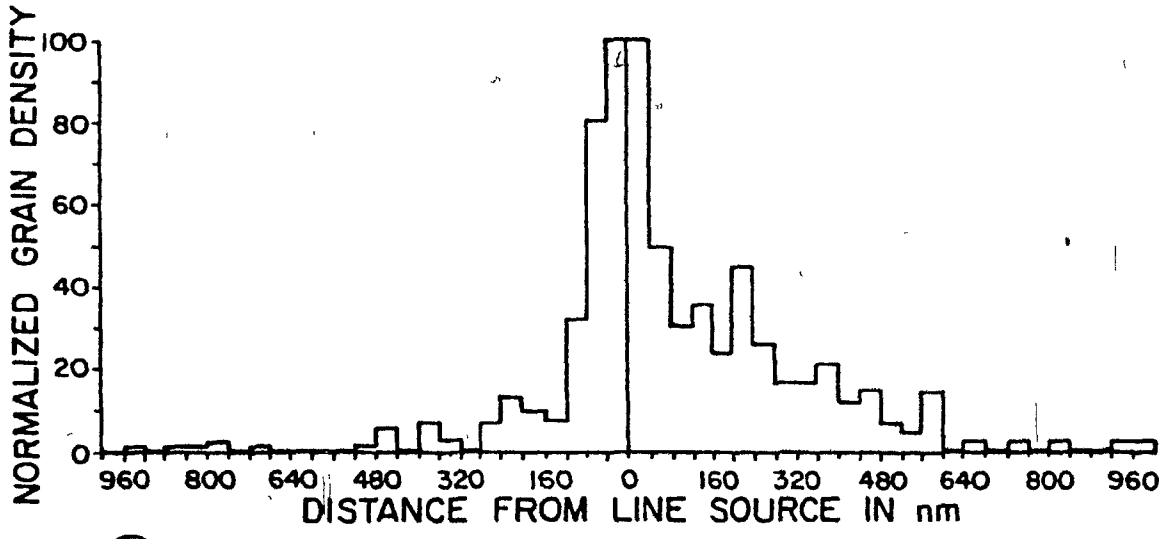
Figures I-12 - I-16 These histograms portray the normalized distributions of grains (i.e., maximum = 100%) seen over the "Epon" and "celloidin" sides of the line source, in relation to their unit distance away from the line. The left-hand curve indicates the pattern over the celloidin side, while the right-hand graph shows the grain distribution over the Epon side of the line.

Figure I-12 This graph portrays the silver grain distribution around a ^{3}H line source section, dipped in Ilford L4 emulsion and developed with Kodak D19b. The use of a χ^2 test for observed values ($p = 0.05$) indicated no significant difference between the left and right sides of the curve. This is interpreted to show that self-absorption by the 100 nm thick Epon section was negligible.

Figure I-13 This histogram shows the normalized distribution for a ^{3}H line source, dipped in Ilford L4 and developed with GSPD producing fine grains. In spite of the apparent difference observed visually, no significant difference was found for data on either side of the line by a χ^2 test for observed values ($p = 0.05$). The shape of the curve indicates a possible decrease in resolution due to some self-absorption by Epon, but this was negligible.


NORMALIZED GRAIN DENSITY
100
80
60
40
20
0
960 800 640 480 320 160 0 160 320 480 640 800 960
DISTANCE FROM LINE SOURCE IN nm

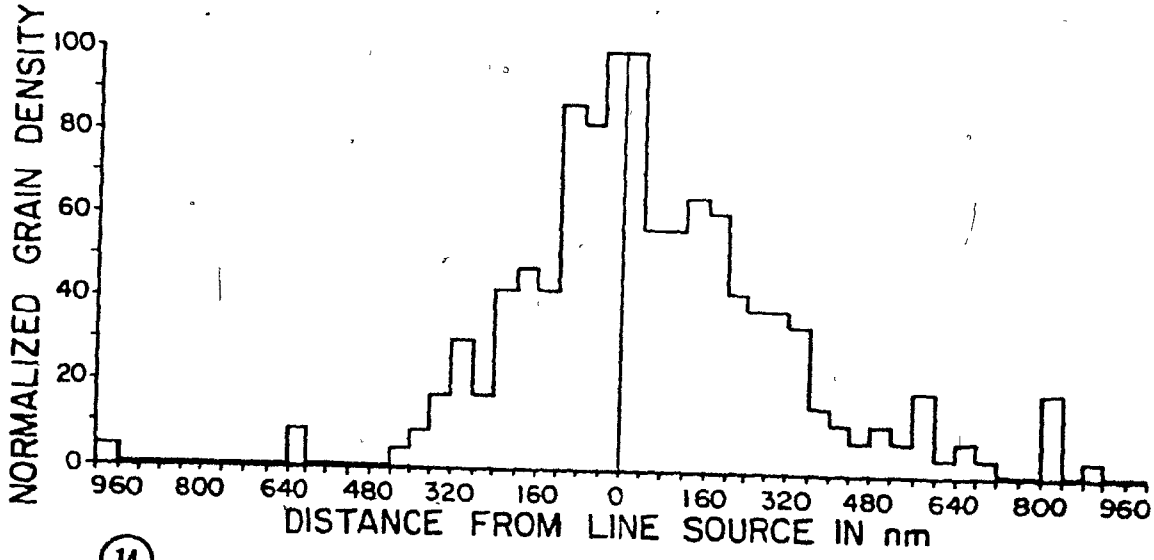
(12)


NORMALIZED GRAIN DENSITY
100
80
60
40
20
0
960 800 640 480 320 160 0 160 320 480 640 800 960
DISTANCE FROM LINE SOURCE IN nm

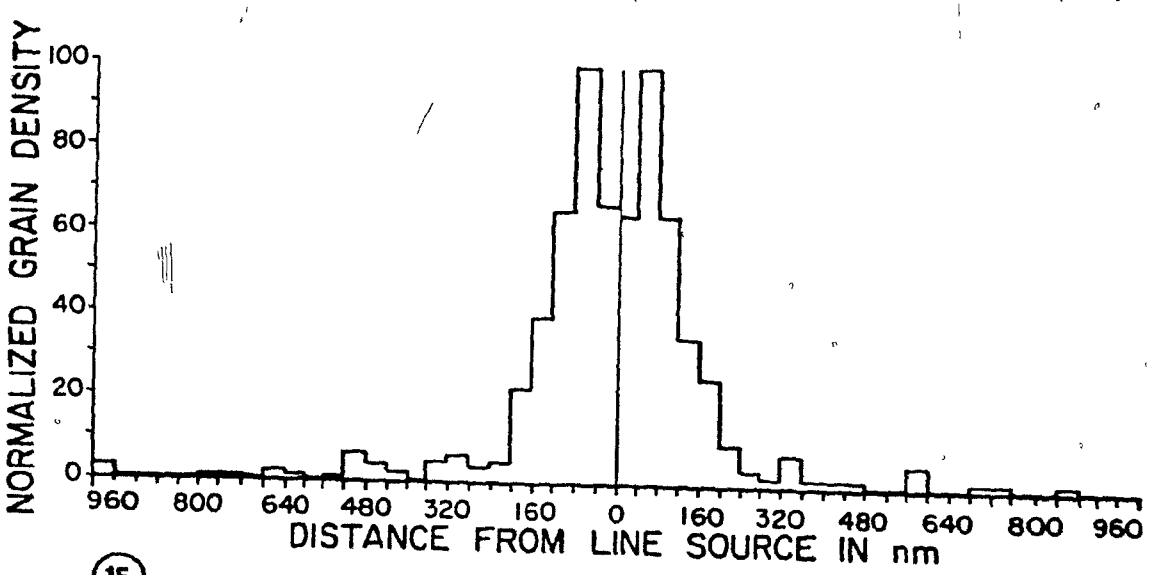
(13)

Figure I-14 A histogram of the normalized grain distribution on either side of a ^{125}I line source is shown. The radioautographs were exposed after dipping in Ilford L4 emulsion and were then developed in Kodak D19b. The silver grains formed were of the filamentous type. No significant ($p = 0.05$) difference was found by χ^2 between normalized grain counts per unit distance from the source, over the Epon and celloidin sides of the line.

Figure I-15 This graph represents the distribution of fine grains produced by the solution physical development with GSPD, for an ^{125}I resolution specimen dipped in Ilford L4 emulsion. The pattern of grains over the celloidin and Epon sides are almost mirror-images of one another. Statistically, they were not found to be significantly different by χ^2 where $p = 0.05$.

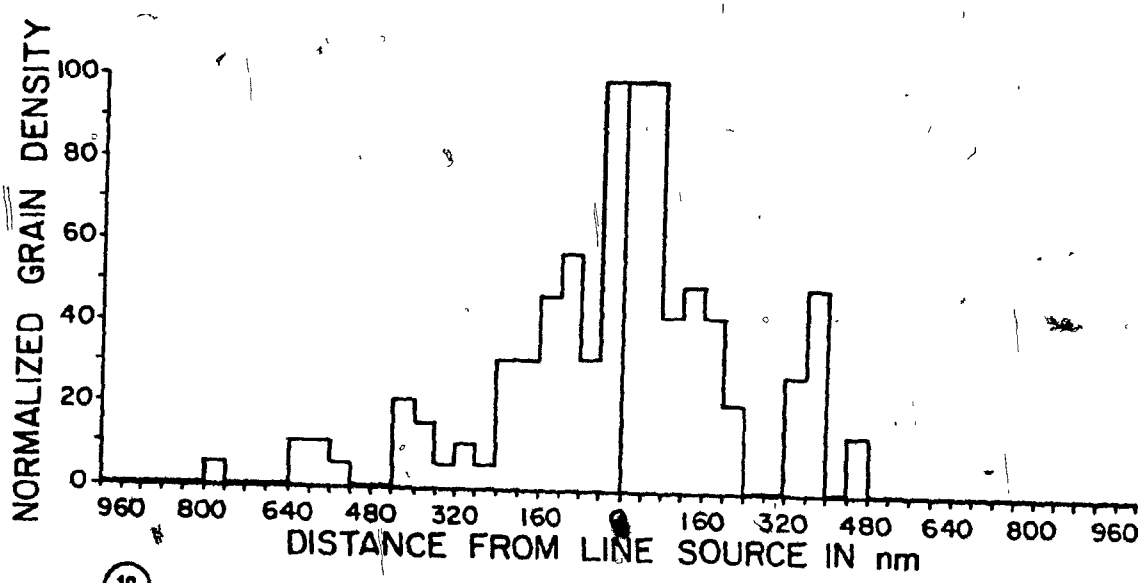

NORMALIZED GRAIN DENSITY
100
80
60
40
20
0
960 800 640 480 320 160 0 160 320 480 640 800 960
DISTANCE FROM LINE SOURCE IN nm

(14)


NORMALIZED GRAIN DENSITY
100
80
60
40
20
0
960 800 640 480 320 160 0 160 320 480 640 800 960
DISTANCE FROM LINE SOURCE IN nm

(15)

Figure I-16 This figure demonstrates the distribution of grains over Epon and celloidin sides of a ^{3}H line source, dipped in Kodak NTE emulsion (with a silver bromide crystal diameter of 0.05 μm). The radioautograph was developed with D19b. The grain produced was a small irregular type of filament. Although there was a significant difference between normalized grain counts on either side of the line, no difference was found between the actual grain counts. For the latter reason, results from the two sides were accumulated to form one-sided displacements, as with the previously shown specimens.


NORMALIZED GRAIN DENSITY
100
80
60
40
20
0
960
800
640
480
320
160
160
320
480
640
800
960
DISTANCE FROM LINE SOURCE IN nm

(16)

INTRODUCTION - PARTS II AND III

At about the same time as the development of the Salpeter method, various analytical techniques were offered by several investigators. Some were alternative approaches, producing similar results to those of Salpeter. Others provided the additional information of relative content and concentration of label within ultrastructural compartments.

The following summaries describe the more notable of these quantitative methods in order to demonstrate the procedures available for radioautographic analysis at the electron microscope level.

1) *History*

a) *The Method of Whur (Whur et al., 1969)*

This method was devised in order to eliminate spurious grain counts which might be attributed to non-labelled structures, due to their proximity to labelled ones. Since a β-ray (or any energy particle) may travel more than 1 μm from a radiation source, it does not necessarily hit a silver bromide crystal directly overlying the source. Even when this does occur, the single crystal or developed silver grain can overlap several structures (Whur *et al.*, 1969). In fact, many of the grains in E.M. radioautographs could be attributed to more than one structure if the assessment is made by visual analysis alone.

To overcome this, a circle with a radius equivalent to 225 nm was drawn around the assumed centre of the developed silver grain. Based on calculations by N. J. Nadler, this yielded a 95% probability circle and was in agreement with results shown by Caro (1962), using a ^{3}H-labelled 60 nm bacteriophage particle as the radioactive source.

Circles with a radius of 6.7 mm for a magnification of 30,000X were drawn on clear plastic sheets. Structures within the circles superimposed over grains were identified, and a scoring of points was implemented whereby a single structure which occupied the entire circle was awarded 1 point. When 2, 3, 4 or more structures were seen within the circle, each structure received 1/2, 1/3, 1/4 or correspondingly less points respectively. The number of points for a given structure was expressed as a percentage of the total grains counted. After counting approximately 200 grains, values were obtained for a category called "uncorrected grain counts". In similar fashion, values for what was termed "random grain distribution" were assessed by superimposing 500 randomly distributed 95% probability circles over the micrographs, and again applying the system of awarding points. The difference between the random and uncorrected distributions yielded values for "corrected grain counts", and only those reactions which were different from random were considered significant. The value for corrected grains reflected neither actual grain concentration nor actual radioactivity, but indicated only where a reaction could be attributed to a particular structure. From these data, further information could be derived to demonstrate the mean concentration of label as a ratio of one organelle to another. Relative concentration could be derived by the ratio of uncorrected grain counts to the relative area occupied by this structure.

Criticism

Although the derivation of relative content of label was considered an advance over the method of Salpeter (1969), the theoretical drawbacks of this technique posed major problems.

The procedure of awarding points to structures as described, resulted in points being scored on the basis of equal partition of grains shared between structures, instead of the more accurate scoring of points according to the probability each structure had of having caused that grain to form. This could have affected the interpretation of grain distributions such that they would not reflect actual grain distributions. As well, final results could act only as estimates of the true concentrations due to this discrepancy. Since the correction factor for random grain distribution was based on the random distribution of structures, this could also have created errors, especially in the case of organelles which were less randomly distributed than others, for example, the Golgi apparatus as compared to rough endoplasmic reticulum (Whur *et al.*, 1969).

Therefore, this method did not actually provide an accurate assessment of silver grain distributions. It could be used to demonstrate where a radioautographic reaction was significant, but only estimates of content and concentration values could be predicted.

b) The Method of Williams (1969)

This method of grain analysis consisted of four sequential operations. The first was the collection of data. This was performed by drawing around the assumed centre of the silver grain a "resolution equivalent diameter" circle, which would take into account the range of the potential isotope source. This range was based on the 50% probability circle of Salpeter (1969), but any arbitrary probability and thus circle size could have been used. The nomenclature followed that where one structure alone occupied the entire circle, this was termed a single entity. Where the circle touched on two or more structures, the term junctional entities were used to describe

both. Where two or more structures fit entirely within the circle, these were considered as compound entities.

The second step in the analysis was in relation to the areas and organization of potential isotope sources. By superimposition of random circles equivalent to silver grains, what was called "effective area" measurement was carried out. Circles equivalent to the appropriate probability were drawn on clear plastic sheets. The "effective area" measurements were made by including at least 200 circles on 16" x 20" paper, in either random or regular arrays, such that the area taken up by the circles reflected the diffusivity or dispersion of the structures, and acting as random grain distributions could then be compared to actual grain distributions.

The third step was to make "actual area" measurements by means of a point analysis whereby a point was drawn at the centre of the circles used for effective area measurement. An alternative approach was to place seven hexagonally arranged points within each circle. From this, relative or actual areas occupied by certain structures could be assessed.

The fourth step concerned the collation of data obtained in the last three operations. The random circle frequency corresponded to a random grain distribution and, therefore, by comparing actual silver grain distributions to the distribution of random circles by a χ^2 test for expected and observed values, the non-random character of the incorporation of label could be assessed.

Further to this, once a non-random character of radioactive label was established, the density of grains per unit area could be used as a measure of radioactivity per unit volume.

However, this approach did not take into account the grains over junctional or compound regions. The following formula was applied to grain counts, in order to allow the study of grains over junctional regions, that is, over more than one structure.

$$G_{AB} = \frac{G_{AA}}{A_{AA}} \times A_{AB} + \frac{G_{BB}}{A_{BB}} \times A_{BA}$$

where G_{AB} = grains over AB

G_{AA} = grains over A

G_{BB} = grains over B

A_{AA} = effective area of A

A_{BB} = effective area of B

A_{AB} = circles with centre on A, but overlap B

A_{BA} = circles with centre on B, but overlap A.

Thus, a prediction of expected grains over junctional regions could be derived from the values for exclusive grains.

For grains over compound regions, a test was applied whereby the effect of including compound regions as possible sources of radiation was compared with results established by including only one of the compound structures alone. That is, if structures A and C are included in the region entirely underlying a grain, the effect of scoring only A, or only C, as the assumed source, was compared to scoring AC as compound items.

The advantages offered by this technique included, according to Williams (1969):

1) a consistent and objective approach

2) analysis of grains "astride" two or more items

3) analysis of items smaller than resolution boundary circle size

4) an approximate measure of resolution.

Criticism

One of the major disadvantages of this method was the laborious collection of data. More than this, however, was the drawback that this method was not quantitative in that it did not provide data as to relative quantities of radioactivity within tissue compartments. Rather, it could be used only to determine the location of a significant radioautographic reaction.

The technique was "as yet imperfect" (Williams, 1969), but did indicate the direction needed for a truly quantitative approach to electron microscope radioautography.

c) *The Method of Nadler (1971)*

The object of the method of Nadler was to assign, by statistical analysis, each silver grain in an electron microscope radioautograph to individual structures in the tissue. This posed two problems.

The first was to establish a relationship between the location of the grain and the source which caused it. To solve this, theoretical probabilities of grain distributions were calculated and a circle of a size corresponding to an arbitrarily chosen probability was drawn around the centre of each silver grain. This resolution boundary circle contained within it all structures which might possibly have been the radioactive source which caused the grain to form. For the method of Nadler, the size of the resolution boundary circle was based upon theoretical considerations. However, the manner by which the size is derived is not essential to the actual method of analysis which is contained in the second problem.

The second problem was to assign each grain within its resolution boundary circle to either one individual structure (exclusive grain = $_nE$), or to more than one structure (shared grain = $_nS$). If all grains were exclusive, there would be little or no problem in interpreting the location of the grain. However, as long as there were shared grains, a problem of interpretation existed. Since it was a fair assumption that for low energy particles, one radioactive emission would produce only one grain (Nadler, 1951; Pelc, 1963, Fertuck & Salpeter, 1974), then only one of the structures "shared" by the grain could have been the true source of radiation.

In practice, the method required an unbiased selection of photographs of fields in the electron microscope which showed at least one grain. A significant number of grains (approximately 500) had to be counted. The goal of the analysis was to attribute to each class of structures, such as mitochondria, nucleus, Golgi, rER, etc., a *true* grain count (= $_nC$; equivalent to "corrected", "predicted" or "expected" count, as opposed to "observed" or "real" count), where the total number of grains enumerated, $T_{EM} = \Sigma\ _nC$. Since shared grains were counted more than once, $T_{GRAND} > T_{EM}$.

The proportion of grains shared by classes n, n', n'', which could be attributed to n alone, was expressed by:

$$\frac{_nC}{_nC + _{n'}C + _{n''}C}$$

Application of a point-hit analysis (Chalkley, 1943) was made in order to determine the relative areas of structures in the radioautographs.

In order to correct for grains shared as a result of the diffuse or compact shape of an organelle, application of a circle-hit analysis (Nadler, 1971) was made as well. Point-hits reflected the area occupied by a structure and circle-hits reflected the chance that the structure would be caught by a circle of that size. The ratio of point-hits to circle-hits is an expression of the chance of a structure being caught by the circles, relative to the area occupied by that structure. Structures which are diffuse as opposed to compact will more likely have a higher ratio, where compact structures will be reflected by a lower ratio. Since diffuse structures will also be more likely to be caught by the resolution boundary circles, then the same ratio will correct for the relative "diffusivity" of the structures.

Point-hits over structure n, expressed as a percentage of the total number of points was designated as ${}_{n}Vp$. Circle-hits, expressed as above, were designated by ${}_{n}Vo$. The ratio of ${}_{n}Vp/{}_{n}Vo$ was regarded as the index of diffusivity of structure n.

If the number of grains shared by n, n', n'' = ${}_{n}sn'$; and if the number of grains attributed to n alone ${}_{n}Sn'$, n'' ; therefore, by equation 1:

$$1)\quad {}_{n}Sn', n'' = {}_{n}sn', n'' \times \frac{{}_{n}C\,({}_{n}Vp/{}_{n}Vo)}{{}_{n}C({}_{n}Vp/{}_{n}Vo) + {}_{n'}C({}_{n'}Vp/{}_{n'}Vo) + {}_{n''}C({}_{n''}Vp/{}_{n''}Vo)}$$

At this point, the value for C was still unknown, but Equation 2 yielded:

$$2)\quad {}_{n}C = {}_{n}E + \Sigma_{n}S$$

Then, Equation 3 stated:

$$3) \quad {}_{n}C_{1} = {}_{n}E \cdot \frac{T_{EM}}{\Sigma_{n} E}$$

By numerical analysis, the sequence from Equation 3 was repeated, with the aid of a Hewlett-Packard electronic calculator (9810A, Model 10), programmed for the necessary calculations, until the value for ${}_{n}C_{k}$ differed by no more than 5% from ${}_{n}C_{k-1}$. When this outcome was reached, ${}_{n}C_{k}$ was accepted as the value for the true grain count ${}_{n}C$.

With this method, determination of relative content of label was made by calculating ${}_{n}C/T_{EM}$ X 100, that is, the ratio of true grain counts over a structure to the total grains enumerated and expressed as a percentage of the total.

In addition, relative concentration of label could be calculated, by $\frac{{}_{n}C/T_{EM}}{{}_{n}Vp}$, that is, the ratio of relative content of label over a structure to the relative volume (area) of that structure, expressed as a percentage of the total volumes.

d) The Method of Blackett & Parry (1973)

By combining an adaptation to the circle analysis of Williams, and other mathematical considerations and manipulations, the method of Blackett & Parry provided an estimate of activity or content of radioactive label within structures seen in electron microscope radioautographs. The method also took into account the geometric shape of organelles and their positions within tissue sections.

The method involved analysing experimentally observed grain distributions around a resolution specimen (line source). From this, the appropriate corresponding distance was calculated for a point source of

radioactivity (Salpeter *et al.*, 1969, 1977). Then using a resolution boundary circle of a size reflecting the chosen probability range, hypothetical grain distributions were determined (Blackett & Parry, 1973). These hypothetical grain distributions were then compared to real grain distributions and the activities were arbitrarily altered until a statistically significant good fit was obtained between them, using the χ^2 test for comparing expected and observed values.

The distributions of hypothetical grains was based on a uniform grid of points on transparent acetate sheets constituting the hypothetical sources of radioactive disintegrations. A list of various "distances" and "directions" were applied to these points, based on probabilities of a point source producing a grain at any given distance from the source. Thus, an analysis similar to the circle analysis of Williams (1969) was made. A circle of radius equivalent to HR for a point source (half-radius; Salpeter *et al.*, 1969), was drawn around the site of each hypothetical grain.

The comparison of real grain to hypothetical grain distributions was made with the aid of a computer program which incorporated a minimising sub-routine (Blackett & Parry, 1977) and explored a range of activities which might have been consistent with a statistically good fit. Once the best fit was determined, the relative activity (content) and the relative area for each organelle was derived. From this data, the relative concentration of label could then be calculated, based on the ratio of relative activity to relative area.

ii) *Problems to be Investigated*

a) *Objective of Parts II and III*

The objective of these studies was to compare the two main analytical methods available for quantitative electron microscope radioautography, and hopefully to draw conclusions concerning the relative merits of both. Moreover, each method depends on a resolution boundary circle which is based on either theoretical analysis or on experimental observations which still require theoretical manipulations. Accordingly, these two quantitative analyses were also compared using a relatively large radius resolution boundary circle of 230 nm, a small size circle of 100 nm, and a nil resolution boundary circle of 0 nm, in order to assess the importance of the size of the resolution boundary circle for the practical interpretation of silver grain distributions in electron microscope radioautographs.

b) *Objective of Part II*

The quantitative analysis of Nadler (1971) and that of Blackett & Parry (1973) was applied to electron microscope radioautographs of thyroid tissue, four hours after injection of $^{55}FeCl_3$, in order to compare the techniques using experimentally observed grain distributions as a substrate for the test system.

c) *Objective of Part III*

The methods of Nadler and Blackett & Parry were also applied to the quantitative analysis of theoretical cell models, in the form of specific hypothetical tissue standards (see Materials and Methods), following the introduction of a hypothetical isotope.

Since the Blackett & Parry method was based on hypothetical grain

distributions which originate from an assumed uniform distribution of radioactivity, the hypothetical grain distributions used in these studies were also based on the assumption that the radio-isotope was uniformly distributed. Thus, it was possible again to analyse the effects of varying the resolution boundary circle radius, from 0 nm to 100 nm and to 230 nm, this time under various hypothetical conditions.

PART II

MATERIALS AND METHODS

Comparison of Techniques used in Assessing Grain Distributions in Electron Microscope Radioautography, Based on Studies of an Experimental Situation

A. *Preparation of Tissue*

(Acknowledgements to Dr. S. Cassol, for supplying the tissue and E.M. radioautographs.)

In experiments previously conducted by Dr. S. Cassol (1975), formerly of this Department, two male Sherman rats, weighing about 100 grams, were slowly injected through the external jugular vein with 0.91 mCi of $FeCl_3$ in 0.18 ml of 0.001 N HCl (carrier free, New England Nuclear Corp., Boston, Mass.). Animals were sacrificed 4 hours after the injection, by sequential perfusion through the left ventricle, first with 3% p-formaldehyde for 15 minutes, followed by 2.5% glutaraldehyde for another 15 minutes. The thyroid glands were removed and immersed in the same fixative for 1½ hours at 4°C, during which time the glands were chopped into small pieces. These were post-fixed in 1% osmium tetroxide in 0.1 M Sörensen's phosphate buffer for 2 hours at 4°C, dehydrated in acetone and embedded in Epon.

B. *Preparation of Electron Microscope Radioautographs*

The tissues were treated by Dr. Cassol in the following manner. Silver-gold sections were cut and transferred to glass microscope slides, coated with 0.8% celloidin. These slides were then dipped in dilute Ilford L4 emulsion with a semi-automatic device, to ensure a single, uniform monolayer of closely packed silver bromide crystals (Kopriwa, 1967).

After exposure times ranging from 76 days to 17 months at 4°C, the radioautographs were developed with Kodak D19b, diluted 1:10, for 1 minute at 20°C. Radioautographs were subsequently fixed for 2 minutes in 24% sodium thiosulfate (Fisher Scientific Co., Montreal, Canada). Thin sections were post-stained for 45 seconds with aqueous uranyl acetate (Stempak and Ward, 1964) followed by 20 minutes with lead citrate (Reynolds, 1963).

C. *Quantitative Electron Microscope Radioautography*

Specimens were placed, by Dr. Cassol, in the electron microscope and all regions showing silver grains were photographed without bias; that is, no portion of a thyroid follicle, whether cell or lumen, which exhibited silver grains was excluded. Approximately 150 pictures, showing about 230 silver grains were taken at the 4 hour time interval. Since the distribution of grains was similar in each pair of the animals studied, grains of both animals were added together.

After having obtained these photographs from Dr. Cassol, quantitation was carried out on these 8" X 10" prints of the electron micrographs, at a final magnification of 30,000X (see Fig. II-5).

i) Quantitation According to the Method of Nadler (Appendix to Haddad et al., 1971)

With the quantitative analysis of Nadler, each silver grain was circumscribed within a resolution boundary circle of either 230 nm, which was equivalent to a 95% probability circle (Whur *et al.*, 1969), or within a circle of 100 nm radius, as a separate study. The structures within this circle were recorded and the grains were scored as either "exclusive",

over only one structure, or as "shared", that is over two or more organelles. Grains were scored over all structures, but for classes where the number of exclusive grains was small (less than 1% of the total), these counts were included in another closely related class which showed a significant number of silver grains. For example, in the final analysis, since so few grains were seen over dense bodies, these were designated as grains over lysosomes. Grains over the Golgi apparatus were not distinguished as being over saccules or vesicles, but instead over the Golgi region. Similarly, attempts were not made to assign grains over the rough endoplasmic reticulum, to the cisternae, membranes or ribosomes, but rather to the rER as a whole. Therefore, the final data tabulated included grains classified as either exclusive to an organelle or shared over more than one of the following: mitochondria, apical vesicles, rER, nucleus, Golgi, colloid, lysosomes and plasma membrane.

In addition to the two different circle sizes analysed, the situation in which a nil resolution boundary circle (one of 0 nm radius) was also investigated. For this, the geometric centre of the developed silver grain was taken as the exclusive site of radioactivity. Therefore no grains were designated as shared, thus eliminating the need for any further application of statistical formulae to this data.

The relative volumes of the various organelles were assessed by the "point-hit" method (Chalkley, 1943; Nadler, 1971), and expressed as a percentage of the total number of circles applied to the photographs. Once the corrected or true grain counts had been established, according to the formula for the solution of a system of non-linear equations, (Nadler, 1971; Nadler and Chajut, 1975), the relative content and relative concentration

of label in each organelle could be determined. In order to derive the corrected counts, the recorded data for exclusive and shared grains, as well as the data concerning point-hits and circle-hits, were fed into an electronic computer (Hewlett-Packard Model 10, 9810A; Hewlett-Packard, Montreal, Canada), programmed to perform the necessary calculations (Nadler, 1971) and print the output data of corrected or true grain counts as exclusive grains over each of the organelles (see Figs. II-1 to 3).

Due to limitations in the computer program, which can presently accommodate only five variables (organelles) at a time, it was necessary to organize the data in such a way that the corrected grain counts, predicted by the computer program, would be independent of the particular combination of structures included in a single run of the program. Since there were eight different organelles to be analysed in this study, eight different combinations of the structures, in groups of five at a time, were run through the computer program. That is to say, that if there were structures labelled A, B, C, D, E, F, G, H, and they were analysed in groups of five, the corrected counts for, let us say organelle B, based on grouping A, B, C, D, E, might be different from those calculated from grouping B, C, D, E, F, especially where all counts are inter-dependent due to grains being shared over more than one structure within or outside of that unique five variable group. If grains are shared between A and B, but A is not included in a particular group, then the corrected counts over B might differ from from one group to the next. The final values for corrected counts which showed the greatest agreement between like organelles from group to group were accepted as the definitive true counts (see Fig. II-A).

ii) Quantitation According to the Method of Blackett & Parry using Hypothetical Grain Distributions (Blackett & Parry, 1973)

This technique for assessing "true" grain distributions involved superimposing transparencies of hypothetical sources and sites of radioactivity over organelles in electron micrograph prints of radioautographs. Transparencies were prepared according to the format of Blackett & Parry (1973). A grid of uniform points (sources of radiation) was printed on 8" X 10" acetate sheets and a series of computer-generated random angles and distances were allocated to all the points. These "new" positions, at which each angle and distance coincided, became the centre of the hypothetical site of radiation. This, in practice, was a circle of specific diameter. The effects of two different circle sizes were analysed. These included a large circle of 230 nm radius and a small circle of 100 nm radius (see Fig. II-4).

The situation in which a nil resolution boundary circle of 0 nm radius was studied as well, this being under the same conditions as described for the method of Nadler. Here, the site of radioactivity, taken as the geometric centre of the silver grain, was assumed to be the exclusive source of radioactivity. In this case, since there were no shared grains, the necessity of any further analysis was eliminated.

By superimposing these transparencies over each of the 8" X 10" photographs, it was possible to derive a table of distribution of hypothetical sources and sites of radioactivity. Approximately 3,000 hypothetical grains were analysed for each circle size.

The hypothetical distributions were then correlated to the real (observed) grain distribution using the χ^2 test for significance, and

indicated the "goodness of fit" of the theoretical data to that of the observed. From this information relative activity or content of label within organelles was determined, as well as the relative areas of each organelle. This was accomplished with the aid of a computer program (which incorporates a minimising sub-routine) designed specifically for this purpose by the Blackett and Parry group.

iii) Comparison of Results Derived from both Quantitative Methods

In order to compare the information gathered using both quantitative techniques, it was necessary to translate the final data from each into units or relative content and relative concentration of radioactive label. The methods were then reviewed in terms of their similarities and differences, as well as their relation to the potential importance of the size of the resolution boundary circle.

Statistical comparisons of the data were carried out by means of a χ^2 test for two sets of observed frequencies (Freund, 1962), as described in Part I of this thesis. The tests were run using a program for the Hewlett-Packard 9810A electronic calculator.

RESULTS - PART II

Experimentally Observed Grain Distributions Assessed by the Methods of Nadler and Blackett & Parry with Varying Circle Sizes

Table II-1 demonstrates the distribution of relative content of radioactive label from radioautographs of thyroid follicles, four hours after injection of $^{55}FeCl_3$ (kindly supplied by Dr. S. A. Cassol). The use of a nil resolution boundary circle (0 nm) and both the 100 nm and 230 nm circles was made by the analysis of data by both the method of Nadler as well as that of Blackett & Parry. Relative content or activity is expressed as a percentage of the total grains counted.

A) Grain Distribution over Organelles

I. Effects of Methods and Circle Size

1) Nucleus, Colloid, Lysosomes, Golgi

Grain counts over the nucleus (∿9%), colloid (∿9%), lysosomes (∿2-3%) and Golgi (∿2-3%), were consistent regardless of the method or circle size used.

2) Rough Endoplasmic Reticulum

It was noted that counts over the rER, derived by using a nil resolution boundary circle (35.5%) were close to those assessed with the 230 nm circle of Blackett & Parry method (32.1 ± 5.7%). The remaining values for the rER, derived with the 100 nm circle for the method of Blackett & Parry, and the 100 nm and 230 nm circles for the method of Nadler, ranged from 45% to about 70%.

3) *Apical Vesicles*

Counts over the apical vesicles were consistent at about 5 to 7% for the 0 nm circle and both circle sizes with the method of Nadler. However, those derived by the method of Blackett & Parry, for both circle sizes, were lower at about 0 to 2%.

4) *Basal and Lateral Membranes*

Values over the basal and lateral membranes were consistent at about 13% for the 0 nm circle and for both circle sizes with the method of Blackett & Parry. In this case though, the method of Nadler estimated lower values of about 5 to 8%.

5) *Mitochondria*

Most striking of all organelles were the differences between percentages predicted for grain counts over the mitochondria, depending on the circle size or method of quantitation used. With the nil resolution boundary circle, about 17% of the grains were attributed to this organelle. The method of Nadler, using a 100 nm circle, accords 12% of the label to mitochondria, while using the 230 nm circle, 26% of the label is given to the mitochondria.

The Blackett & Parry method predicts a comparatively low value of 3.4%, with a large standard error of ±12.3%, when using the 100 nm circle. At the same time this method, using a 230 nm circle, attributed 31.4 ± 3.4% of all grains to mitochondria.

B) *Comparison by χ^2 of Grain Distributions Assessed by Both Methods and Circle Sizes*

Comparisons of the predicted distributions were made using a χ^2 test for two sets of observed data (Freund, J. E., 1962). Results are shown in

Table II-2. The distributions of relative content were compared regarding results derived with the 0 nm circle to those of both the 100 nm and 230 nm circles and also for the methods of Nadler and Blackett & Parry. Comparisons were also made between the two methods and between the 100 nm and 200 nm circle sizes. The results of these tests were dependent upon a confidence level of 95% ($p = 0.05$), which was accepted as statistically significant.

The following is a sample calculation of χ^2 for a comparison between the 0 nm circle and the 230 nm circle for the method of Nadler:

$$\chi^2 = \frac{N}{N_A} \cdot \left[\frac{a_1^2}{N_1} + \frac{a_2^2}{N_2} + \cdots + \frac{a_k^2}{N_k}\right] + \frac{N}{N_B} \cdot \left[\frac{b_1^2}{N_1} + \frac{b_2^2}{N_2} + \cdots \frac{b_k^2}{N_k}\right] - N$$

$$= \frac{200}{100} \cdot \left[\frac{35.5^2}{80.9} + \frac{9.2^2}{18.1} + \frac{9.7^2}{18.1} + \frac{7.9^2}{12.8} + \frac{17.1^2}{43.0} + \frac{3.1^2}{4.6} + \frac{4.0^2}{4.4} + \frac{13.6^2}{18.2}\right]$$

$$+ \frac{200}{100} \cdot \left[\frac{45.4^2}{80.9} + \frac{8.9^2}{18.1} + \frac{8.4^2}{18.1} + \frac{4.9^2}{12.8} + \frac{25.9^2}{43.0} + \frac{1.5^2}{4.6} + \frac{0.4^2}{4.4} + \frac{4.6^2}{18.2}\right]$$

$$- 200$$

$$= 106.08 + 105.85 - 200 = \underline{11.93}$$

Since 11.93 is less than the value of 14.06 predicted for the χ^2 distribution for 7 degrees of freedom, where $p = 0.05$, therefore the difference between the two groups of data was not significant.

All χ^2 tests were executed by a computer program for the Hewlett-Packard electronic calculator, 9810, Model 10.

1) *The Method of Nadler in Relation to Varying Circle Size*

Using the method of Nadler, no significant difference was observed between results derived from the 0 nm circle, when compared to those found using either the 100 nm or 230 nm circle. In addition, there was no significant difference between results derived with the 100 nm and 230 nm circles when compared to each other.

2) *The Method of Blackett & Parry in Relation to Varying Circle Size*

There was no significant difference between results, using the 0 nm circle and those established with a 230 nm circle. However, there were significant differences between results from the 0 nm and 100 nm circles, as well as between the 100 nm and 230 nm circles.

3) *Comparison of Both Methods in Relation to Varying Circle Size*

Results found with the 100 nm and 230 nm circles used with the method of Nadler were compared to those found with the same circle sizes respectively for the method of Blackett & Parry. No significant difference was found between the methods when the circle sizes compared with the same, that is, 100 nm to 100 nm, and 230 nm to 230 nm. However, there were significant differences when comparing the 100 nm circle used with the method of Nadler to the 230 nm circle used with the Blackett & Parry method. As well, there were significant differences when comparing the 230 nm circle of the Nadler method to the 100 nm circle of the Blackett & Parry method.

TABLE II-1

Distribution of Relative Content of Label as determined using the Methods of Nadler and Blackett & Parry for 0 nm, 100 nm & 230 nm resolution boundary circle

Relative Content

*Expressed as percentage of total**

		Blackett & Parry ± S.E.M.		Nadler	
Organelle	0 nm Circle	100 nm	230 nm	100 nm	230 nm
rEr	35.5	65.8 ± 18.6	32.1 ± 5.7	52.0	45.4
Nucleus	9.2	9.0 ± 1.7	9.2 ± 3.4	9.6	8.9
Colloid	9.7	8.8 ± 3.9	8.0 ± 3.6	9.5	8.4
Apical Vesicles	7.9	1.1 ± 0.8	0.7 ± 1.3	6.9	4.9
Mitochondria	17.1	3.4 ± 12.3	31.4 ± 3.4	11.5	25.9
Lysosomes	3.1	0.6 ± 2.0	3.9 ± 3.8	1.2	1.5
Golgi	4.0	2.2 ± 1.4	0.9 ± 2.8	2.1	0.4
Basal & Lateral Membranes	13.6	9.0 ± 4.3	13.8 ± 4.0	7.6	4.6

* Percentages may not add up to exactly 100, due to rounding.

TABLE II-2

Results of χ^2 Comparison of Methods and Circle Sizes

χ^2 Comparison of Method	Circle Size	to Method	Circle Size	χ^2 Significance* ($p = 0.05$)
Direct Count	0 nm	Nadler	100 nm	n.s.
Direct Count	0 nm	Nadler	230 nm	n.s.
Nadler	100 nm	Nadler	230 nm	n.s.
Direct Count	0 nm	B&P	100 nm	s.
Direct Count	0 nm	B&P	230 nm	n.s.
B&P	100 nm	B&P	230 nm	s.
B&P	100 nm	Nadler	100 nm	n.s.
B&P	230 nm	Nadler	230 nm	n.s.
B&P	100 nm	Nadler	230 nm	s.
B&P	230 nm	Nadler	100 nm	s.

* n.s. = not significantly different

s. = significantly different

Figure Legends - Part II

Figure II-A This is a sample of the program print-out from the Hewlett-Packard (9810-A) desk-top calculator, showing the input data prior to analysis by the method of Nadler, and the output data of *true* grain counts after such analysis. The data shown is for a resolution boundary circle of 100 nm radius. Due to limitations in the present computer program, only 5 variables can be accommodated at a time, thus necessitating analysis for more than five organelles, by including at least two different groups of 5, as shown for 8 variables in this case.

Input

Exclusive Grains

Nucleus	18.69
rER	77.60+
Mitochondria	13.69-
Golgi	4.00+
Membranes	14.09+

Shared Grains

N+R	6.69+
N+G	0.00+
R+M	28.69-
R+G	2.00+
R+Mb	7.00+
G+Mb	0.00+
N+R+G	0.00+
R+G+Mb	0.00+

Volumes

N Vp	17.30+
N Vo	16.20+
R Vp	46.60+
R Vo	44.40-
M Vp	3.50+
M Vo	1.30+
G Vp	4.10+
G Vo	3.00+
Mb Vp	5.80+
Mb Vo	4.70+

Output

T_{EM}	169.00
Loop Number	3.00+
Sensitivity(p)	0.05
Iterations	545.00

TRUE COUNTS

Nucleus	18.69
rER	108.22
Mitochondria	22.83
Golgi	4.10
Membranes	14.96
± S.D.	0.01

Computation Time

Hours	0.00
Minutes	5.00
Seconds	36.00

End Print

Input

Exclusive Grains

Colloid	17.69-
rER	77.30+
Lysosomes	2.50+
Ap. Vesicles	11.00+
Membranes	14.00+

Shared Grains

C+R	1.00+
C+Av	3.00+
R+L	2.90+
R+Av	7.90+
R+Mb	7.90+
Av+Mb	0.00+
C+R+Av	0.00+
R+Av+Mb	0.00+

Volumes

C Vp	18.70+
C Vo	18.30+
R Vp	46.60+
R Vo	44.40+
L Vp	1.70+
L Vo	0.70+
Av Vp	2.40+
Av Vo	1.70+
Mb Vp	5.80+
Mb Vo	4.70+

Output

T_{EM}	147.00
Loop Number	3.00+
Sensitivity(p)	0.05
Iterations	545.00

TRUE COUNTS

Colloid	18.65
rER	97.22
Lysosomes	2.44
Ap. Vesicles	13.62
Membranes	15.07
± S.D.	0.01

Computation Time

Hours	0.00
Minutes	5.09
Seconds	36.00

End Print

Ⓐ

Figure II-B. This is a sample of the computer print-out supplied after analysis by the method of Blackett & Parry, which incorporates a minimising subroutine. The distribution of hypothetical sites and sources of radio-activity are seen under the heading "Original Grains." Real grains (RG) are compared with hypothetical grains (HG) and the best χ^2 fit is determined, as shown in the lower rows of data. Results derived are for a resolution boundary circle of 230 mm radius.

ENTER (MAN)UAL,AUTØ ØR (FIX)ED X·
? auto
14 X STARTING VALUES
? 1,1,1,1,1,1,1,1,1,1,1,1,1,1

NKN	SLK	IPRINT	ICØN	MAXIT	ESCALE	E
10	3	0	1	200	200	·100LCAL

ØRIGINAL GRAINS

SITE	R	N	C	Av	M	L	G	Mb	R+N	R+M	R+G	R+Mb	R+L	C+Av
rER	750	14	6	1	0	0	5	10	51	190	22	121	62	4
Nucleus	9	375	0	0	0	0	0	1	60	7	2	1	2	1
Colloid	2	0	451	3	0	0	0	0	0	4	0	1	0	36
Ap. Vesicles	4	0	6	24	0	0	0	1	0	1	1	0	2	14
Mitochondria	15	2	0	3	2	0	2	1	2	57	1	2	2	1
Lysosomes	12	1	0	0	0	4	1	0	0	1	0	0	21	0
Golgi	18	2	0	0	0	0	57	0	0	2	24	1	0	0
Membranes	41	3	0	0	0	1	0	25	2	3	1	73	3	0
RG	54	17	15	7	1	1	1	5	9	54	6	23	10	3
HG	62	18	15	3	1	*1	3	6	7	52	3	22	10	3
X2	1·0	·1	·0	0·	0·	0·	0·	·2	·6	·0	0·	·1	·0	0·

TØTAL CHI SQUARED 2·0 WITH 7 DEGREES ØF FREEDØM

COMPUTER FIT WITH ESTIMATED STD· ERRØRS

SØURCE	GRAINS/GRID PØINT	REL· ACTIVITY	REL· AREA
rER	·053 ± ·011	32·1 5·7	47·0 ± ·8
Nucleus	·041 ± ·014	9·2 ± 3·4	17·4 ± ·7
Colloid	·033 ± ·014	8·0 ± 3·6	18·9 ± ·6
Apical Vesicles	·029 ± ·061	·7 ± 1·3	2·0 ± ·3
Mitochondria	·719 ± ·096	31·4 ± 3·4	3·4 ± ·4
Lysosomes	·203 ± ·206	3·9 ± 3·8	1·5 ± ·3
Golgi	·019 ± ·067	·9 ± 2·8	4·0 ± ·4
Membranes	·187 ± ·051	13·8 ± 4·0	5·8 ± ·5

STD· ERRØR ØF CHI SQD· 5·3

(B)

Figure Legends - Part II

Figures II-1 to II-5. Electron microscope radioautoraphs of rat thyroid follicular cells, four hours after injection of $^{55}FeCl_3$ (kindly supplied by Dr. S. A. Cassol, formerly of this Department). X 30,000

N = nucleus
M = mitochondria
G = Golgi
rER = Rough endoplasmic reticulum
lcm = Lateral cell membrane
av = Apical vesicles
mv = Microvilli adjacent to colloid

Note silver grains over nucleus, rER, lateral cell membrane and microvillar region.

Figures II-1 to II-3. With transparencies superimposed for the method of Nadler.

Figure II-4. With transparency superimposed for the method of Blackett & Parry.

Figure II-1. Point-hit transparency superimposed over radioautograph to assess relative areas of organelles.

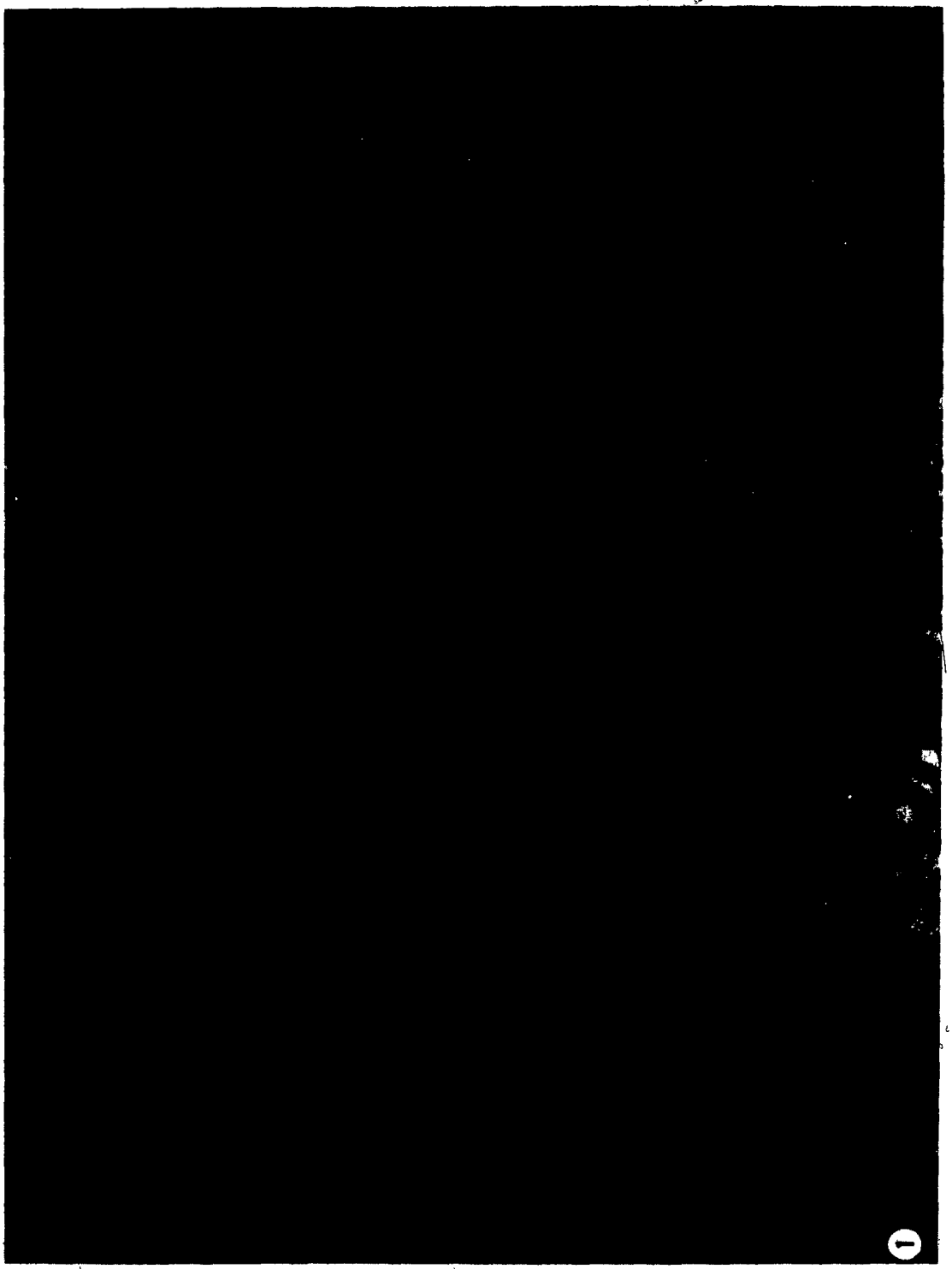


Figure II-2. Circle-hit transparency superimposed over radioautograph to assess relative diffusivity of organelles. Circles are equivalent to resolution boundary circles of 230 nm radius.

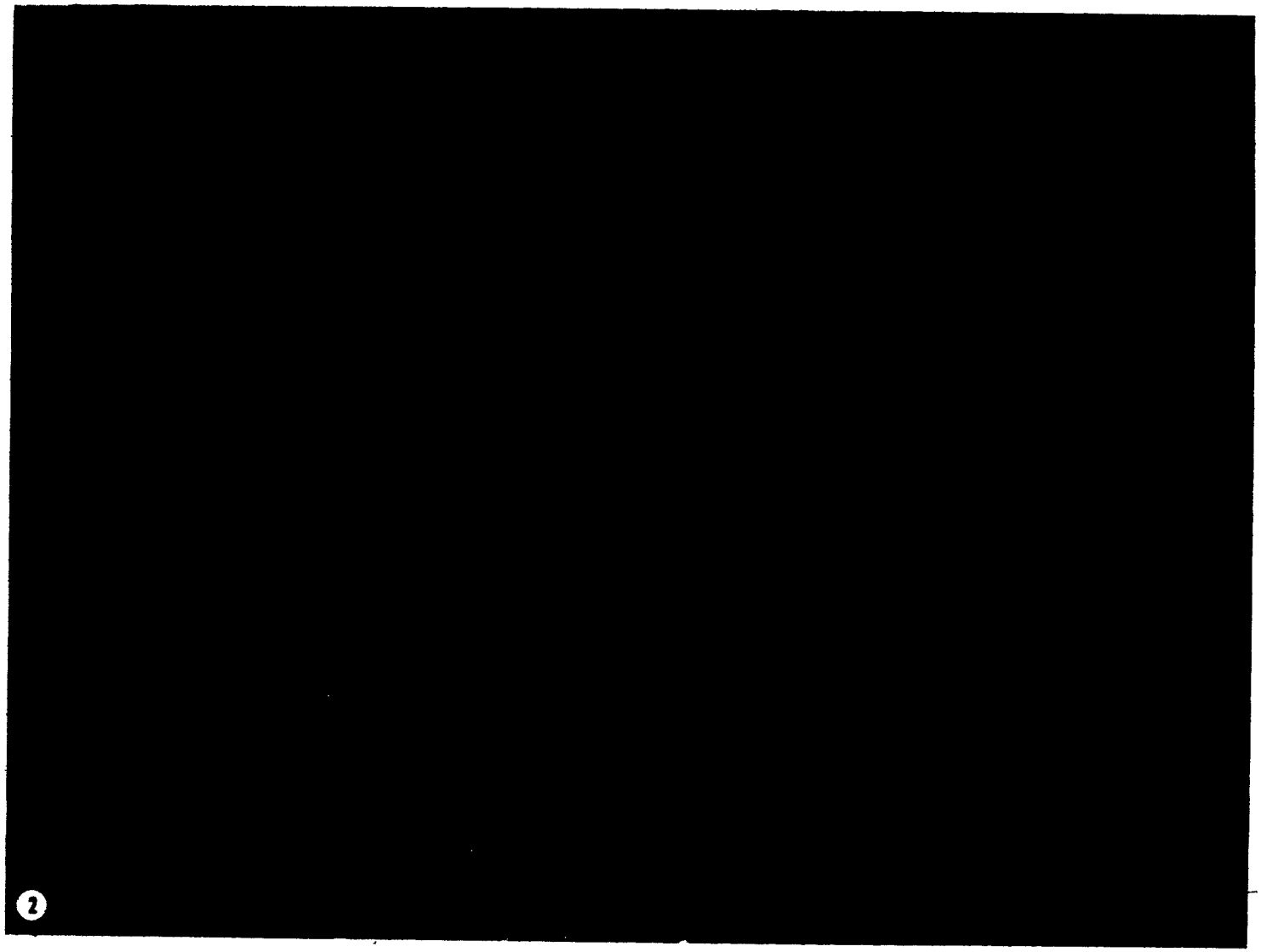

2

Figure II-3. Circle-hit transparency superimposed over radioautograph. Circles are equivalent to resolution boundary circles of 100 nm.

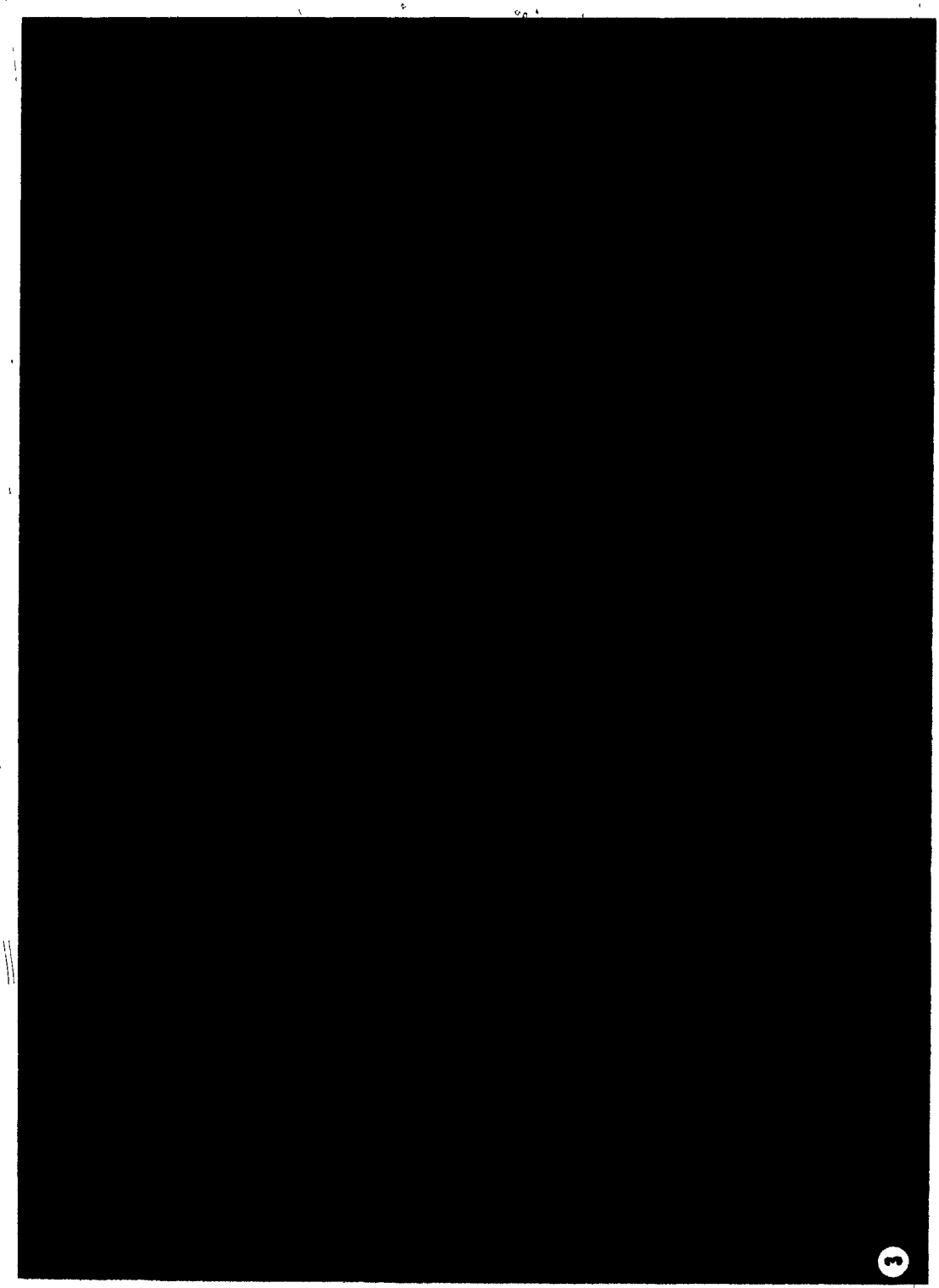


Figure II-4. Transparency of the form suggested by Blackett & Parry (1973) superimposed over radioautograph. It consists of a uniform distribution of points (hypothetical radioactive sources), from which are derived sites of hypothetical grains, circumscribed by both a 100 nm and 230 nm radius resolution boundary circle.

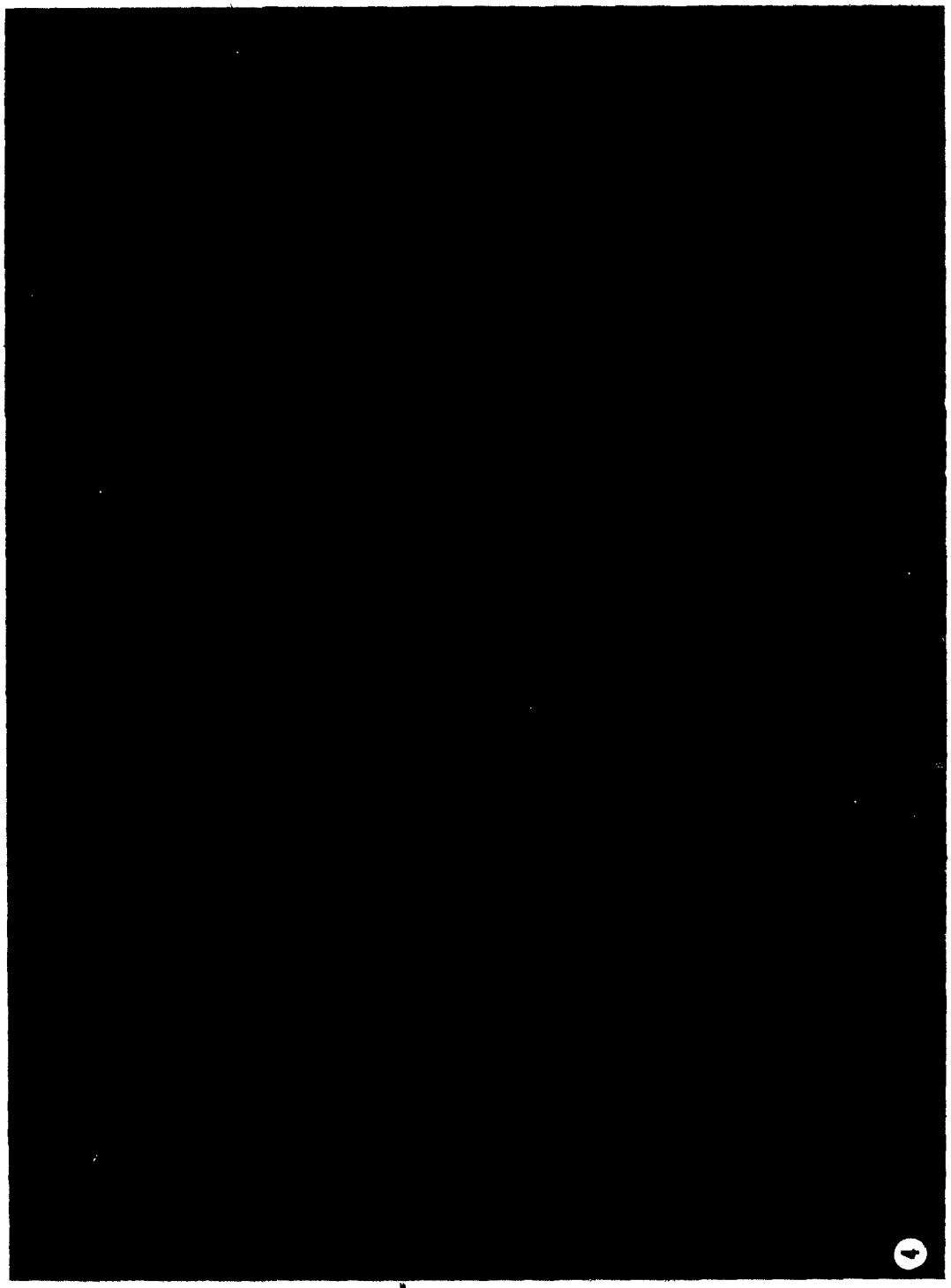
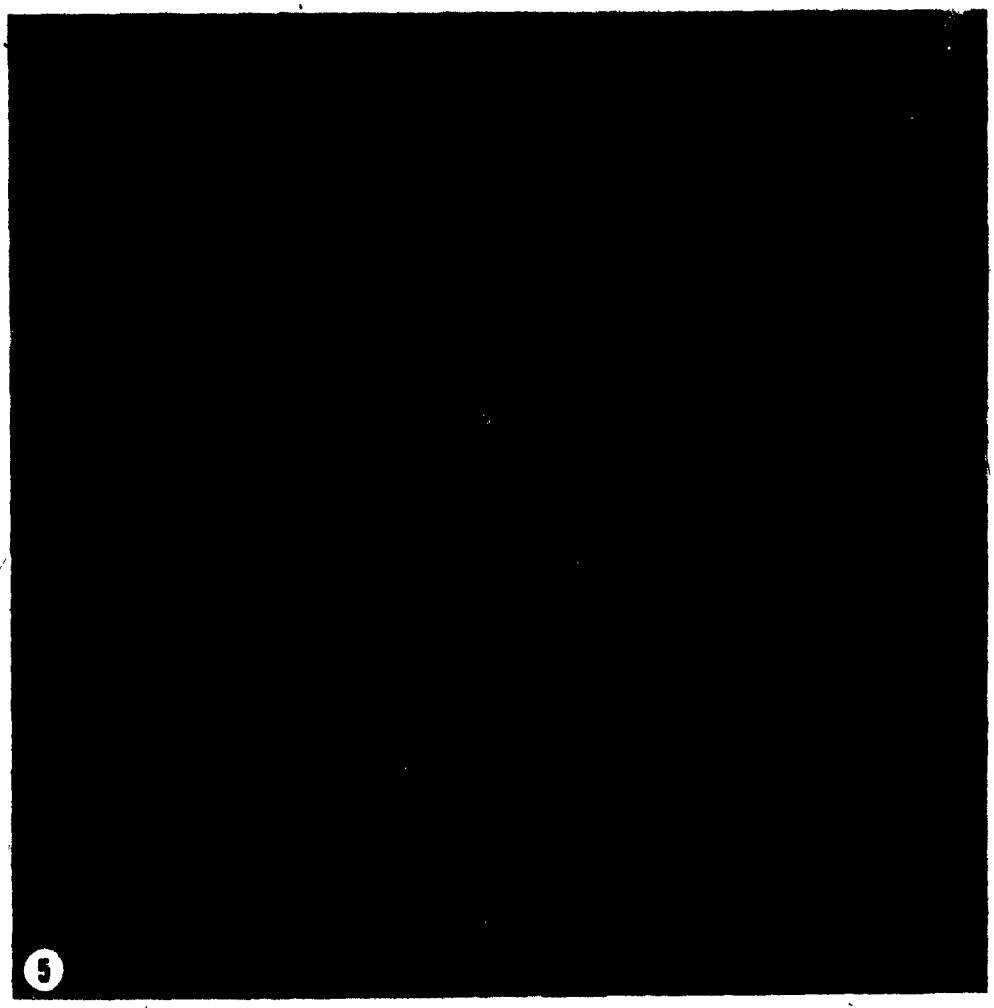


Figure II-5. This shows an example of the problem encountered in determining the true source of radioactivity from electron microscope radioautographs. Arrow indicates filamentous type of grain shared by more than one organelle in the tissue section. Possible locations of the radioactive source which produced this grain include:- mitochondria, rER, cytosol, lateral cell membrane and perhaps Golgi.


5

PART III

MATERIALS AND METHODS

Comparison of Techniques used in Assessing Grain Distributions in Electron Microscope Radioautography, Based on Studies of Hypothetical Tissue Standards

In this analysis, the quantitative method of Nadler (1971) was studied in relation to that of Blackett & Parry (1973), using "Hypothetical Tissue Standards", prepared with the aid of photography and computer generated random numbers and angles, as described in the following procedures.

A. *Preparation of Tissue*

The thyroid glands and liver tissue of male Sherman rats were chosen for the construction of hypothetical tissue standards.

(Acknowledgements to Dr. S. A. Cassol, Dr. J. J. M. Bergeron and Mr. G. Batky for providing electron micrographs of these tissues.)

Animals were sacrificed by intra-cardiac perfusion of 2.5% glutaraldehyde in buffer. Tissues were treated for routine electron microscopy and embedded in Epon. Thin sections were cut and post-stained with uranyl acetate and lead citrate.

B. *Preparation of Hypothetical Tissue Standard Composite Photographs*

Electron micrographs were analysed, the photographic images of specific examples of organelles were chosen, and then cut out of the paper with scissors (nucleus, mitochondria, rER, Golgi). The "cut-out" images of these organelles were then re-photographed with an Asahi Pentax 35 mm Single Lens Reflex camera and the developed prints processed at various specific magnifications (5 different magnifications for the nucleus; 3

different magnifications for other organelles), in order to produce various sizes of the same organelle images (see Table III-A). These images were then cut out of the photographic paper, as before, with scissors. They were then placed on a blank paper background, according to the following conditions.

The background was placed over a 100 X 100 line, 2 dimensional matrix grid, numbered from 1-100 on both the X and Y axes (on 'Albanene' guideline prepared tracing paper; Keuffel & Esser, 11" X 17", Cat. No. 10-5663). This, in turn, was placed over a standard fluorescent X-ray viewer, positioned horizontally on a desk-top platform. With the X-ray viewer illuminated, the grid lines could be visualized projecting through the blank paper background overlying them. With the fluorescent screen turned off, the grid lines disappeared, and with the aid of overhead lights the image of the clear background was illuminated. By using a set of computer generated random numbers (in co-ordinate pairs), and random angles, the cut-out images of organelles were placed in position on the background, adhering to these conditions. Each organelle was assigned a specific polarity (A-B), such that Point A was always the reference for the placement of the structure on the grid line background, according to the co-ordinate pairs of random numbers generated. Keeping Point A fixed, Point B was rotated according to the random angles generated by the computer. (The horizontal axis of the photograph was designated as 180° or 'East-West'.)

If a position was already occupied by a previously placed organelle, the numbers associated with that area were eliminated and new values for random placement were chosen. Thus, no two structures could overlap each other, but could rest closely together, as is found with organelles seen

in actual electron micrographs.

In this way, a series of 33 different composite photographs was prepared, by altering from one to the next, the sizes of the organelles, the quantity of the same organelles and their random (predicted by computer) positions within the confines of the 8" X 10" area assigned as the background cytosol (see Table III-B). Each separate composite hypothetical tissue standard was then photographed with an Asahi Pentax 35 mm SLR camera and the developed print was enlarged on photographic paper so that the final composite photograph would be the same size as it appeared in the original layout (i.e. 8" X 10") and thus, size comparisons could be made with actual electron micrographs for investigative study (see Figures 1-33), at equivalent magnification.

C. *Preparation of Transparencies Necessary for Quantitation*

1) *Quantitation by the Method of Nadler (1971).*

For the quantitative analysis of Nadler, point-hit and circle-hit transparencies were used to determine the relative volumes and dispersion of organelles within the composite photographs. Separate transparencies were also used to include uniform arrays of 100 circles with radii equivalent to either 100 nm or 230 nm. The circles on these transparencies acted as resolution boundary circles, and structures within them were recorded as if there were a silver grain within each circle. In other words, each circle was assumed to circumscribe a grain, based on the rationale that an "ideal" uniform incorporation of radioactivity into all cellular compartments would produce an "ideal" uniform display of silver grains (Nadler, 1971; Blackett and Parry, 1973).

The distribution of exclusive and shared "grains" was scored for each composite and this information, in conjunction with the point-hits and circle-hits, was assessed for both circle sizes, and for every individual composite tissue standard. The corrected (true) grain counts were evaluated according to the formula for the solution of a system of non-linear equations (Nadler, 1971), as previously described in Part II C(i). In this case, since only five organelles were analysed, it was necessary to run the computer program only once for each composite.

Under the circumstance where a nil resolution boundary circle (0 nm) was considered, "grain" counts were designated by scoring the structure directly underlying the centre of the circle on the transparency which, in effect, was the same as scoring the point-hits as "grain" counts.

Since relative concentration of label is equivalent to the ratio of percentage grain counts to percentage area, then the relative concentration, established by using a 0 nm resolution boundary circle, was equivalent to 1.00.

ii) Quantitation by the Method of Blackett and Parry (1973)

For the quantitative analysis of Blackett and Parry, transparencies were prepared according to the format required, as described in Part II C(ii). With these transparencies, the distribution of hypothetical sources and sites of radioactivity was determined for each of the hypothetical tissue standard composites. The designation of "real grains" was made by using a grid of uniformly distributed circles (100), with radii equivalent to either 100 nm, or 230 nm, in exactly the same manner as "real" or "observed" grains were determined in the analysis of the method of Nadler (Part III C(i)).

iii) Comparison of Results Derived from both Quantitative Methods

The results of both techniques, being the 'corrected' or 'true' results, were translated into units of relative activity or content and relative concentration of 'label' over organelles in the composites, in order to determine the similarities and differences in the methods when put into practice. The importance of the resolution boundary circle size, and in addition potential deficiencies of one method as opposed to the other, were evaluated.

Based on the assumption of uniform activity throughout the tissue composite, the "ideal" concentration value was expressed as unity (1.00). Therefore, the corrected results were compared to uniform activity and expressed as a percentage of variation from this value (Tables III-3 and 4) Where applicable, statistical comparisons were also made, using the χ^2 test of significance for two sets of observed data (Freund, 1962).

Tables and graphs were organized from this information and demonstrated for comparative investigation (see Tables III-1 to 12a, Fig. III-A, B).

TABLE IIIA

Areas of Organelles used in Construction of Hypothetical Tissue Standard Composites

Organelle	Size (X)	Avg. Area cm^2	± SD	Area Ratios
NUCLEUS	½ X	12.15	.05	0.25
	1 X	48.30	0	1
	1½ X	105.27	.70	2.18
	2 X	187.78	.38	3.89
	2½ X	295.80	.78	6.12
MITOCHONDRIA	½ X	0.42	.10	0.14
	1 X	3.03	.06	1
	2 X	11.13	.20	3.67
ROUGH ER	½ X	12.98	.33	0.23
	1 X	57.77	.61	1
	2 X	205.47	1.37	3.56
GOLGI	½ X	2.07	.12	0.25
	1 X	8.20	.10	1
	2 X	31.47	.15	3.84
CYTOSOL	-	508.95	-	-

* Areas were assessed by planimetry (Hirsch, Zelickson and Hartmann, 1965), and expressed as the average of 3 readings.

The 1 X size was the original size as seen in the electron micrograph from which organelles were chosen.

For the nucleus, this magnification was 12,750X.

For other organelles, it was 30,000X.

TABLE IIIB

Sizes and Quantities of Organelles Positioned Randomly within Cytosol in Hypothetical Tissue Standard Composites

Composite Number	Organelles							
	NUCLEUS		ROUGH ER		GOLGI		MITOCHONDRIA	
	Size(X)	Q.*	Size	Q.	Size	Q.	Size	Q.
1	½ X	1	1 X	1	1 X	1	1 X	10
2	½ X	2	"		"		"	
3	1 X	1	"		"		"	
4	1 X	2	"		"		"	
5	1½ X	1	"		"		"	
6	2 X	1	"		"		"	
7	2½ X	1	"		"		"	
8	1 X	1	½ X	1	1 X	1	1 X	10
9	"		½ X	2	"		"	
10	"		1 X	2	"		"	
11	"		2 X	1	"		"	
12	1 X	1	1 X	1	½ X	1	1 X	10
13	"		"		½ X	2	"	
14	"		"		1 X	2	"	
15	"		"		2 X	1	"	
16	"		"		2 X	2	"	

(continued)

TABLE IIIB (continued)

Sizes and Quantities of Organelles Positioned Randomly within Cytosol in Hypothetical Tissue Standard Composites

Composite Number	Organelles							
	NUCLEUS		ROUGH ER		GOLGI		MITOCHONDRIA	
	Size(X)	Q.*	Size	Q.	Size	Q.	Size	Q.
17	1 X	1	1 X	1	1 X	1	½ X	5
18	"		"		"		½ X	10
19	"		"		"		½ X	20
20	"		"		"		1 X	5
**21	"		"		"		1 X	10
22	"		"		"		1 X	20
23	"		"		"		2 X	5
24	"		"		"		2 X	10
25	"		"		"		2 X	20
26	1 X	2	1 X	2	1 X	2	1 X	10
27	1 X	2	1 X	2	1 X	2	1 X	20
28	½ X	1	½ X	1	½ X	1	½ X	10
29	½ x	2	½ X	2	½ X	2	½ X	20
30	2½ X	1	1 X	1	1 X	1	½ X	20
31	½ X	1	½ X	1	½ X	1	2 X	20
32	½ X	1	½ X	1	2 X	1	½ X	20
33	½ X	1	2 X	1	½ X	1	½ X	10

*Q. = Quantity

**#21 - same organelles as #3, but different positions.

RESULTS - PART III

The methods of quantitation and the sizes of the resolution boundary circle were analysed using hypothetical tissue standards in the form of composite photographs, as seen in Figures III-1 to III-33. Data is given in Tables III-1 to III-12a (also see sample print-out Fig. III-C, D).

Results demonstrating the relative content of label as determined using the method of Nadler for the 0 nm, 100 nm and 230 nm circle sizes, are shown in Table III-1.

Results for the method of Blackett & Parry are shown in Table III-2.

A) *Comparison of Relative Concentration of Label*

I. *Variation from Unit Concentration*

Relative concentration of label was determined by the ratio of relative content of label in an organelle to the relative area of that organelle. Since there was an assumed uniform distribution of label, relative content ideally should have been the same as relative area for all organelles. Thus, comparisons of the data derived by the two methods and circle sizes could be made on the basis of the observed variations from the assumed unit concentration. Table III-3 shows a comparison of the averages of variations of the "corrected" or "expected" data from unit concentration (1.00), expressed as percentages. That is, a concentration value of 1.50, for example, would have been a variation of 50% from unit concentration. In order to derive the average variations, variation of all 'like' organelles from all composites (i.e., rER from composites 1-33, nucleus from composites 1-33, etc...) were determined, such that the values in Table III-3 represent the averages of all variations for each organelle of all the composite hypothetical tissue standards.

The average percent variations from unit concentration did not differ markedly on preliminary inspection. Detailed analysis was made using the χ^2 test of significance for two sets of observed values (Freund, 1962). A confidence level of 95% ($p = 0.05$) was chosen as statistically significant. The results of these tests are shown in Table III-4.

1. *Average of Variations All Organelles included*

a) *Method of Nadler - Comparison of Circle Sizes*

Results derived from both sizes of resolution boundary circle showed no significant difference between them. The data from the 230 nm circle however, did show a consistently greater variation from unit concentration than that of the 100 nm circle.

b) *Method of Blackett & Parry - Comparison of Circle Sizes*

The average percentage variations from unit concentration were not significantly different in reference to the comparison of data derived using either the 100 nm or 230 nm circles.

c) *Comparison of the Method of Nadler to that of Blackett & Parry*

There was no significant difference found between results derived with either the 100 nm circles from both analytical methods, or between the 230 nm circles from both methods. Moreover, cross-reference comparisons of the 100 nm circle of one method to the 230 nm circle of the other method showed no significant difference between average percent variations from unit concentration. When considering all five organelles, the average percent variations of the five collectively were, for the Blackett & Parry method, using the 100 nm circle, 56.4%, while for the 230 nm circle it was

54.2%. The method of Nadler, using a 100 nm circle, showed an average variation of 37.3%, while for the 230 nm circle it was 56.7%.

2. *Averages of Variations*

(Including organelles 1, 2, 3 only (rER, cytoplasm, nucleus)

As will be discussed in more detail later, apparent differences between some of the results may have been due to the occurrence of relatively low percentage area or low percentage labelling of some of the organelles in these hypothetical tissue standards. This was true especially in the case of the Golgi apparatus and mitochondria (organelles 4 and 5). In many of the composites, these structures account for only 2 to 5% of either the total area or total label. For this reason, minor variations in relative content became vastly increased when converted to percentage variations of concentration. For example, if the relative content of label in the rER was assumed to be 50% but analysis determined it to be 40%, this 10% difference in relative content would be calculated as a 20% variation in relative concentration. A sample calculation follows:

$$\text{Actual variation in concentration} = \frac{0.50}{A} - \frac{0.40}{A} = \frac{0.10}{A} \quad (A = \text{Area})$$

$$\text{Percentage variation} = \frac{0.10}{A} \div \frac{0.50}{A} = \frac{0.10}{A} \times \frac{A}{0.50} = 0.2 \times 100\% =$$

20% variation.

Continuing this rationale, if the relative content of label in the Golgi was assumed to be 2%, but analysis showed the Golgi to contain only 1% of the label, then this actual difference of only 1% is interpreted as a 50% variation from the expected concentration (i.e., 0.02 - 0.01 = 0.01; but 0.01/0.02 = 0.50 X 100% = 50%). Thus, with such small structures, considerably minor variations appeared to be much greater than they actually were.

It was therefore decided to compare the results of both methods and

circle sizes for only the first three organelles in each composite group. By omitting the data for the Golgi and mitochondria, the average percent variations were diminished to about half the original values. As it were, by eliminating these generally small structures with low labelling (about 2 to 5% for both), the average percent variation dropped from 55% down to 30% for the method of Blackett & Parry, and from 47% to 25% for the method of Nadler.

Figures III-A and B are histograms portraying the percentage variation from unit concentration of the results for the rER, cytoplasm and nucleus. The data represent the percentages of individual values exhibiting variation, rather than the averages. The distribution of variations demonstrates that the majority of the values were within a variation of 50% from unit concentration.

3. *Comparison of Underestimates to Overestimates*

As previously described, individual variations were determined in order to compile the data for average percent variations from a uniform distribution of label. For each composite, individual concentration values observed were either equal to, higher or lower than the assumed unit value. Higher values (+) were considered to be overestimates, while lower values (-) were interpreted as underestimates. These data are shown in Table III-5.

a) Method of Blackett & Parry

The use of this method produced approximately equal numbers of overestimates as underestimates on the whole, but not necessarily from within each composite photograph studied. For the 230 nm circle size, about 2% of all predictions were determined precisely as unit concentrations.

b) *Method of Nadler*

The method of Nadler demonstrated a bias for underestimating concentration values. About 70% of all "corrected counts" derived by using a 100 nm circle were less than unit value, while for the 230 nm circle almost 80% of the results were considered as underestimates.

B) Comparison of Relative Content of Label

Tables III-6 to III-12a demonstrate results of the application of the χ^2 test of significance for two sets of observed data. Comparisons were made between both methods and circle sizes. Table III-11 is a summary of the determination by χ^2 of the organelles responsible for differences observed from composite to composite.

I. Comparisons of Individual Composites

1. Method of Nadler

a) Comparison of 100 nm to 230 nm circle

Results of these tests are shown in Table III-6. Where data for "exclusive + shared" grain counts prior to analysis were shown to be different between circle sizes, the exclusive counts alone were not significantly different. Therefore, as would be expected (Nadler, 1971), any differences were attributable to differences in the "shared" grain counts determined by the two differing circle sizes. The "corrected" counts, however, produced after analysis by the method of Nadler, showed no significant differences, regardless of the circle size used for the analysis, for almost all composites, but for one exception. This was seen in Composite 25 (Fig. 25). Further χ^2 analysis, as shown in Table III-6a, determined that the difference was due to organelle 4, the mitochondria.

b) Comparison of the 0 nm circle to the 100 nm and 230 nm circles

Table III-7 shows the results of the χ^2 test of significance for two sets of observed data, on the comparison of "corrected" counts from the 100 nm to 230 nm circle sizes, to those derived using the nil resolution boundary circle.

i) Comparison of the 0 nm to 100 nm circle

The results of the 100 nm circle were significantly different for only one of the thirty-three composites studied. This was Composite 27 (Fig. 27). Further χ^2 analysis (Table III-7b) enabled the determination of the source of difference as having been due to a difference in data for organelle 4, the mitochondria.

ii) Comparison of the 0 nm to 230 nm circle

Results for 19 of the composites listed in Table III-7 displayed significant differences. When additional χ^2 tests were applied to the data for organelles 1, 2 and 3 only (Table III-7a), results from all composites were not significantly different in relation to circle size. The cause of the differences in the results of these analyses was a difference in data between circle sizes for the mitochondria and Golgi.

2. *Method of Blackett & Parry*

Results of χ^2 tests of significance for comparisons of the effects of resolution boundary circle size on the method of analysis are shown in Table III-8.

i) Comparison of the 100 nm to 230 nm circle

Of all the hypothetical tissue standards studied, eight of these differed regarding their assessment by the two circle sizes. Results of further χ^2 testing shown in Table III-8a, allowed the sources of the differences

to be determined, and were due to either the small size and low relative content of label in one or more of the organelles, or to the diffuse distribution of some of the organelles, in particular the mitochondria.

ii) Comparison of the 0 nm to 100 nm circle

In this study, results from five of the composites showed significant differences (Table III-8). The differences were investigated and were found to be due again to the small size of the organelles in question (Composites 1, 2, 12), or as in two of the composites (Figs. 10, 30), to the diffuse extent of an organelle.

iii) Comparison of the 0 nm to 230 nm circle

Significant differences were shown between the nil and 230 nm circle in five of all the composites. Three of these composites (Figs. 1, 2, 30) were the same ones in which differences were seen in the 0 nm to 100 nm circle comparison. Again the cause of the discrepancies appears to be the small relative size and low relative content in organelles, or their dispersion, especially with reference to the mitochondria and Golgi.

3. Comparison of Each Method to the Other

i) Comparison of Results of the 100 nm Circle

Use of the χ^2 test of significance was made. Results from eight of the composites were found to be significantly different (Table III-9). Again, the organelles causing the difference were comparatively small or comparatively diffuse (see Table III-11 and appropriate figures of composites), and the majority were due to mitochondria or Golgi.

ii) Comparison of Results of the 230 nm Circle

Results from 15, or almost half of the composites, showed significant

differences between the methods for the 230 nm circle, as seen in Table III-9. In Table III-9a, results of further χ^2 analyses are shown, which determined the sources of these differences. The mitochondria and Golgi were responsible for differences in 10 of the 15. As well, for the others, it was noted that either the small size or diffuse surface of the organelles were involved in causing variations in the data.

iii) Cross-reference Comparison of the 100 nm Circle (Blackett & Parry) to the 230 nm Circle (Nadler)

Tables III-10 and III-10a show these data, results of the χ^2 test of significance. Where there were differences in sixteen of the composites, their causes were determined to be due mostly to the mitochondria and Golgi, but in general to the smaller size or greater diffusivity of a structure.

iv) Cross-reference Comparison of the 230 nm Circle (Blackett & Parry) to the 100 nm Circle (Nadler)

Once more, referring to Tables III-10, 10a and 11, results are shown of χ^2 analyses. Results from six of the composites displayed significant differences. The sources of the differences were determined. In this case, however, the reasons for the differences were not so apparent, since the causes varied for each composite and were not necessarily attributable to the size or diffusivity of a structure.

II. Average of Relative Content of Label in Organelles from all Composites

The relative contents of like organelles from all composites (i.e., rER from composites 1 to 33, nucleus from composites 1 to 33, etc....) were averaged and the data were compared by χ^2 analysis for differences

between circle sizes and both methods.

Table III-12 shows that results appear to be consistent regardless of circle size or method used. Results by the method of Nadler showed a tendency towards underestimation of smaller structures (less relative area), and overestimation of much larger structures, for example, the cytoplasm. However, as shown in Table III-12a, the differences in total distribution were not significant, as determined by χ^2 ($p = 0.05$).

This was the case for all analyses made, with but one exception. Of the averages compared according to method and circle size, the comparison between the Blackett & Parry method with a 100 nm circle, and the Nadler method with a 230 nm circle displayed a significant difference. Additional investigation attributed the differences to organelles 4 and 5, the mitochondria and the Golgi. Together, these organelles comprised, on the average, only 10% of the total area or label within the hypothetical tissue standards.

TABLE III-1

Data for the Method of Sadler

Relative Content* of Label as determined using a 100 nm and 230 nm Resolution Boundary Circle

Composite No.	Relative Content (%) 100 nm Circle	230 nm Circle	Relative** Area (%)	Composite No.	100 nm Circle	230 nm Circle	Relative Area (%)	Composite No.	100 nm Circle	230 nm Circle	Relative Area (%)
1.	8.5	6.5	11	2.	10.3	5.4	11	3.	6.5	5.6	11
	88.5	92.4	78		85.6	92.5	79		83.3	89.1	72
	1.0	1.0	2		2.0	2.1	3		8.2	5,4	9
	2.1	0.0	7		2.1	0.0	4		1.1	0.0	6
	0.0	0.0	2		0.0	0.0	3		1.0	0.0	2
4.	8.6	4.5	10	5.	8.3	6.6	9	6.	8.5	6.9	9
	74.3	83.9	66		73.0	76.9	65		54.4	62.2	49
	15.0	11.6	17		16.6	15.5	20		33.0	30.9	35
	2.1	0.0	5		1.1	0.0	5		3.2	0.0	6
	0.0	0.0	2		1.0	1.0	1		1.0	0.0	1
7.	6.6	4.9	9	8.	1.0	1.0	2	9.	2.1	0.0	4
	41.7	42.2	33		92.9	92.8	86		89.8	94.7	82
	49.6	52.9	50		6.1	6.2	6		7.2	5.3	9
	1.1	0.0	6		0.0	0.0	4		0.0	0.0	4
	1.1	0.0	2		0.0	0.0	2		1.0	0.0	1
10.	17.4	15.0	21	11.	34.4	33.2	34	12.	8.4	6.6	10
	73.1	79.7	60		58.3	60.6	49		81.3	89.1	75
	7.3	5.4	10		6.2	6.2	8		7.2	4.3	8
	2.2	0.0	8		1.1	0.0	8		3.2	0.0	7
	0.0	0.0	1		0.0	0.0	1		0.0	0.0	0
13.	8.2	6.4	9	14.	8.5	5.5	11	15.	8.2	5.6	10
	82.7	89.5	78		84.3	89.2	72		78.6	85.0	73
	8.1	4.2	6		6.2	5.3	9		8.2	6.3	8
	1.1	0.0	6		1.1	0.0	5		0.0	0.0	4
	0.0	0.0	1		0.0	0.0	3		5.1	3.1	5
16.	8.5	5.6	10	17.	6.3	5.6	10	18.	8.4	7.5	12
	70.5	84.5	62		85.5	89.1	77		83.4	85.2	75
	7.3	5.3	8		7.2	5.4	9		7.2	6.3	8
	2.2	0.0	8		0.0	0.0	2		0.0	0.0	3
	11.5	4.5	12		1.0	0.0	2		1.0	1.0	2
19.	7.5	5.5	11	20.	7.2	4.4	10	21.	8.3	5.5	10
	84.2	88.1	73		81.6	88.2	76		83.5	89.2	78
	6.3	5.4	10		8.2	7.4	10		7.2	5 3	8
	0.0	0.0	4		1.0	0.0	3		0.0	0.0	3
	2.0	1.0	2		0.0	0.0	1		1.0	0.0	1
22.	9.3	6.5	11	23.	8.5	4.6	12	24.	8.3	6.6	11
	78.0	88.2	66		78.9	87.0	68		70.0	78.5	63
	6.3	5.3	9		6.1	6.3	7		8.3	7.3	8
	5.4	0.0	12		5.5	1.2	10		11.5	7.7	16
	1.0	0.0	2		1.1	1.0	3		2.0	0.0	2
25.	7.9	3.5	10	26.	14.5	13.7	18	27.	18.0	12.5	21
	57.6	79.6	48		65.5	74.0	56		64.6	72.4	48
	6.3	6.1	7		14.9	12.4	17		14.1	14.1	15
	28.2	10.5	34		2.1	0.0	6		2.3	0.0	13
	0.0	0.3	1		3.0	0.0	3		1.1	1.0	3
28.	2.0	1.0	2	29.	4.0	1.1	5	30.	8.4	6.8	7
	97.0	98.0	93		92.9	96.9	83		39.4	42.1	37
	1.0	1.0	1		3.1	2.1	4		52.2	51.1	52
	0.0	0.0	3		0.0	0.0	6		0.0	0.0	2
	0.0	0.0	1		0.0	0.0	2		0.0	0.0	2
31.	2.0	0.0	2	32.	2.0	0.0	2	33.	35.0	31.3	37
	84.9	92.7	77		92.9	98.9	85		64.1	67.7	58
	1.0	1.0	2		2.0	0.0	2		1.0	1.0	2
	12.1	6.3	18		0.0	0.0	4		0.0	0.0	2
	0.0	0.0	1		3.1	1.0	7		0.0	0.0	1

* Relative Contents are displayed in all cases for organelles in the following order:

1) rER
2) Cytoplasm
3) Nucleus
4) Mitochondria
5) Golgi

** In these studies *relative area* was equivalent to data for *0 nm* Circle.

TABLE III-2

Data for the Method of Blackett & Parry

Relative Content of Label as determined*

using a 100 nm and 230 nm Resolution Boundary Circle

	Relative Content (%)										
Composite No.	100 nm Circle	230 nm Circle	Relative** Area (%)	Composite No.	100 nm Circle	230 nm Circle	Relative Area (%)	Composite No	100 nm Circle	230 nm Circle	Relative Area (%)
1.	0.0	0.0	0.0	2.	12.3	10.2	8.9	3.	10.7	9.5	9.0
	95.3	93.3	87.8		63.6	81.2	72.8		75.7	75.0	73.2
	4.7	6.0	5.3		0.0	0.0	4.1		9.2	7.2	10.1
	0.0	0.0	4.1		11.5	0.0	8.9		4.4	8.3	7.7
	0.0	0.7	2.8		12.5	8.6	5.3		0.0	0.0	0.0
4.	15.0	13.7	10.2	5.	6.7	6.4	6.5	6.	5.7	14.3	10.2
	62.7	71.3	58.3		61.5	58.8	54.3		53.0	48.0	47.0
	22.3	15.0	21.4		22.1	22.1	19.9		32.9	31.2	32.3
	0.0	0.0	10.2		8.6	12.8	18.7		8.3	6.5	5.3
	0.0	0.0	0.0		1.0	0.0	-0.4		0.1	0.0	5.3
7.	10.7	7.6	10.2	8.	No	0.0	1.6	9.	3.9	0.4	2.8
	37.5	40.4	23.7		print	89.8	85.0		84.7	79.5	77.6
	50.3	52.0	58.3		out	7.5	7.7		6.9	9.9	10.2
	1.6	0.0	7.7			1.0	5.3		4.6	9.0	9.0
	0.0	0.0	0.0			1.0	0.4		1.3	0.3	0.4
10.	50.3	26.1	23.7	11.	34.0	34.9	33.6	12.	8.5	13.3	12.6
	43.3	63.6	58.1		49.7	42.8	48.2		70.9	71.9	75.2
	6.3	9.5	8.9		8.6	18.9	12.5		5.0	6.0	8.9
	1.8	0.0	5.3		7.6	1.0	4.0		3.3	8.8	2.9
	0.8	0.8	4.0		0.3	2.4	1.6		15.7	0.4	0.4
13.	7.6	6.9	11.4	14.	11.8	10.5	7.8	15.	7.6	7.2	5.3
	79.9	71.0	70.4		71.5	81.0	73.1		71.7	71.4	63.0
	10.4	11.9	10.1		4.4	8.5	7.7		8.4	8.0	10.2
	1.1	0.0	4.0		2.3	0.0	11.4		4.6	2.6	11.4
	1.0	0.2	4.0		0.0	0.0	0.0		7.6	10.8	10.2
16.	7.1	11.9	11.5	17.	7.6	12.3	10.2	18.	16.0	14.5	10.1
	58.2	66.0	53.2		83.5	79.0	78.6		65.2	72.0	79.0
	9.9	11.8	8.9		5.7	3.6	7.8		10.1	9.3	5.3
	7.5	6.9	12.6		1.5	0.0	0.4		8.1	3.7	5.2
	17.3	3.5	13.8		1.7	5.1	2.8		0.7	0.6	0.4
19.	8.6	11.9	7.7	20.	3.9	9.8	5.3	21.	15.0	16.6	10.1
	79.6	77.4	77.6		84.9	76.7	87.8		76.8	70.2	70.4
	7.9	9.5	10.2		6.0	5.5	5.3		7.0	9.8	9.0
	1.2	1.5	0.3		5.2	4.0	1.6		7.9	0.0	5.2
	1.1	1.7	0.4		0.0	4.0	0.0		1.1	3.5	5.3
22.	2.8	No	6.5	23.	16.2	13.2	11.4	24.	11.5	9.3	11.4
	81.5	print	67.9		62.4	67.2	64.2		59.9	60.6	59.3
	7.2	out	7.7		10.0	13.3	10.1		8.3	8.7	9.0
	7.0		13.8		11.4	6.2	12.7		17.9	21.0	18.7
	1.6		4.0		0.0	1.0	1.6		2.4	0.3	1.6
25.	2.5	4.9	9.0	26.	12.9	18.7	16.3	27.	15.8	15.1	16.4
	61.1	53.1	21.1		68.7	65.2	54.3		54.3	58.5	48.2
	5.1	5.6	5.3		14.2	16.1	18.8		17.6	19.3	13.8
	29.2	32.3	59.3		0.5	0.5	8.9		12.3	3.7	18.8
	2.0	4.1	5.3		3.7	0.3	1.6		9.9	3.4	2.9
28.				29.	No	2.5	5.3	30.	6.1	14.0	10.2
					print	95.0	91.1		58.3	0.6	27.2
					out	2.5	2.8		35.6	49.6	50.8
	No print out					0.6	0.4		0.0	32.1	10.1
						0.4	0.4		0.7	3.7	1.6
31.	2.5	No	1.6	32.	0.0	0.0	0.0	33.	36.1	40.6	33.6
	92.0	print	80.0		86.8	96.3	92.6		63.9	58.3	59.5
	5.5	out	16.3		2.6	1.7	1.6		0.5	1.1	2.8
	5.8		1.6		9.9	1.8	5.3		0.1	0.0	4.1
	0.3		0.4		0.7	0.2	0.4		0.0	0.7	0.0

* Relative Contents are displayed in all cases for organelles in the following order:

1) rER
2) Cytoplasm
3) Nucleus
4) Mitochondria
5) Golgi

** In these studies *relative area* was equivalent to data for *0 nm*

TABLE III-3

Summary Table of Comparative Analysis of the Quantitative Method of Nadler and of Blackett & Parry

Variation from Unit Concentration

Composite Organelles	Blackett & Parry Method		Nadler Method	
	100 nm circle	230 nm circle	100 nm circle	230 nm circle
	Average % variation from 1.000			
1. rER	42.0	32.3	19.3	45.9
2. Cytoplasm	23.5	21.2	13.2	22.9
3. Nucleus	37.2	24.0	16.0	30.3
4. Mitochondria	81.2	89.5	75.3	95.7
5. Golgi	98.3*	104.2	62.4	88.6
* Excluding value for Golgi from composite 12 due to excessive variation (ca. 4000 %)				
Average of all organelles	56.4	54.2	37.3	56.7
Average of both circle sizes (all organelles)	55.2		47.0	
Average of 1-3; rER + cytoplasm + nucleus (only)	34.2	25.8	16.2	33.0
Average of both circle sizes	30.0		24.6	
Average concentration range (all organelles)	0.44 - 1.56	0.46 - 1.54	0.63 - 1.37	0.43 - 1.57
Average concentration range of 1-3; rER + cytoplasm + nucleus (only)	0.66 - 1.34	0.74 - 1.26	0.83 - 1.16	0.67 - 1.33
Average of both circle sizes	0.70 - 1.30		0.75 - 1.25	

TABLE III-4

Results of χ^2 tests for Comparisons of Average Percent variations from Unit Concentration

Comparison Method	Circle	to Method	Circle	χ^2 Significance ($p = 0.05$)
Nadler	100 nm	Nadler	230 nm	n.s.*
Blackett & Parry (B&P)	100 nm	B&P	230 nm	n.s.
Nadler	100 nm	B&P	100 nm	n.s.
Nadler	230 nm	B&P	230 nm	n.s.
Nadler	100 nm	B&P	230 nm	n.s.
Nadler	230 nm	B&P	100 nm	n.s.

* not significantly different

TABLE III-5

Percentage of Underestimates (-) and Overestimates (+) of Individual Percent Variations from Unit Concentration

Method	Circle Size			
	100 nm		230 nm	
	% (-)	(+)	(-)	(+)
Nadler	70.3	29.7	78.8	21.2
Blackett & Parry	49.3*	50.0	47.2*	50.7*

* Remaining percentage accounted for by values predicted precisely as unit concentration (i.e., no variation).

TABLE III-6

Results of χ^2 Comparisons of Data

Method of Nadler *Comparison of 100 nm to 230 nm circle* *p = 0.05*

Composite No.	Prior to Analysis: Exclusive & Shared Grain Counts	Prior to Analysis: Exclusive Grain Counts only	After Analysis "Corrected" Grain counts
1	n.s.	n.s.	n.s.
2	n.s.	n.s.	n.s.
3	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s.
5	n.s.	n.s.	n.s.
6	s.	n.s.	n.s.
7	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.
9	s.	n.s.	n.s.
10	n.s.	n.s.	n.s.
11	n.s.	n.s.	n.s.
12	n.s.	n.s.	n.s.
13	n.s.	n.s.	n.s.
14	n.s.	n.s.	n.s.
15	s.	n.s.	n.s.
16	s.	n.s.	n.s.
17	n.s.	n.s.	n.s.
18	n.s.	n.s.	n.s.
19	n.s.	n.s.	n.s.
20	n.s.	n.s.	n.s.
21	n.s.	n.s.	n.s.
22	s.	n.s.	n.s.
23	n.s.	n.s.	n.s.
24	n.s.	n.s.	n.s.
25	s.	n.s.	s.
26	s.	n.s.	n.s.
27	s.	n.s.	n.s.
28	n.s.	n.s.	n.s.
29	n.s.	n.s.	n.s.
30	s.	n.s.	n.s.
31	s.	n.s.	n.s.
32	s.	n.s.	n.s.
33	n.s.	n.s.	n.s.

n.s. = not significant

s. = significant

TABLE III-6a

Results of χ^2 Determination of Sources of Difference for Corrected Grain Counts

Composite 25 — *Method of Nadler* — *100 nm to 230 nm circle*

Organelles included	χ^2 Significance (p = 0.05)
1, 2, 3, 4	s.
1, 2, 3, 5	n.s.
1 - 3	n.s.

TABLE III-7

Results of χ^2 Comparisons of Data

Method of Nadler

Comparison of Corrected Grain Counts

derived with 0 nm, 100 nm & 230 nm circle

Comp. No.	Comparison of 0 nm to 100 nm circle	Comparison of 0 nm to 230 nm circle
1	n.s.	s.
2	n.s.	s.
3	n.s.	s.
4	n.s.	s.
5	n.s.	n.s.
6	n.s.	n.s.
7	n.s.	s.
8	n.s.	n.s.
9	n.s.	s.
10	n.s.	s.
11	n.s.	s.
12	n.s.	s.
13	n.s.	n.s.
14	n.s.	s.
15	n.s.	n.s.
16	n.s.	s.
17	n.s.	n.s.
18	n.s.	n.s.
19	n.s.	n.s.
20	n.s.	n.s.
21	n.s.	n.s.
22	n.s.	s.
23	n.s.	s.
24	n.s.	n.s.
25	n.s.	s.
26	n.s.	s.
27	s.	s.
28	n.s.	n.s.
29	n.s.	s.
30	n.s.	n.s.
31	n.s.	s.
32	n.s.	s.
33	n.s.	n.s.

p = 0.05

TABLE III-7a

Results of χ^2 Comparisons of Data

Organelles 1-3 only*

Comp. No.	Comparison of 0 nm to 230 nm circle
1	n.s.
2	n.s.
3	n.s.
4	n.s.
5	n.s.
6	n.s.
7	n.s.
8	n.s.
9	n.s.
10	n.s.
11	n.s.
12	n.s.
13	n.s.
14	n.s.
15	n.s.
16	n.s.
17	n.s.
18	n.s.
19	n.s.
20	n.s.
21	n.s.
22	n.s.
23	n.s.
24	n.s
25	n.s.
26	n.s.
27	n.s.
28	n.s
29	n.s.
30	n.s.
31	n.s.
32	n.s.
33	n.s.

* organelles 1-3 are rER, cytoplasm, nucleus

TABLE III-7b

Results of χ^2 Determination of Sources of Difference for Corrected Grain counts

Composite 27

Method of Nadler

0 nm to 100 nm Circle

Organelles included	χ^2 Significance (p = 0.05)
1, 2, 3, 4	s.
1, 2, 3, 5	n.s.
1 - 3	n.s.

TABLE III-8

Results of χ^2 Comparisons of Data

Method of Blackett & Parry — *Comparison of results for relative content assessed by a 0 nm, 100 nm, 230 nm circle* — $p = 0.05$

Composite No.	Comparison of 0 nm to 100 nm circle	Comparison of 0 nm to 230 nm circle	Comparison of 100 nm to 230 nm circle
1	s.	s.	n.s.
2	s.	s.	s.
3	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s.
5	n.s.	n.s.	n.s.
6	n.s.	n.s.	n.s.
7	n.s.	n.s.	n.s.
8	- *	n.s.	-
9	n.s.	n.s.	n.s.
10	s.	n.s.	s.
11	n.s.	s.	s.
12	s.	n.s.	s.
13	n.s.	n.s.	n.s.
14	n.s.	n.s.	n.s.
15	n.s.	n.s.	n.s.
16	n.s.	n.s.	s.
17	n.s.	n.s.	n.s.
18	n.s.	n.s.	n.s.
19	n.s.	n.s.	n.s.
20	n.s.	n.s.	n.s.
21	n.s.	n.s.	s.
22	n.s.	-	-
23	n.s.	n.s.	n.s.
24	n.s.	n.s.	n.s.
25	n.s.	n.s.	n.s.
26	n.s.	n.s.	n.s.
27	n.s.	n.s.	n.s.
28	-	-	-
29	-	n.s.	-
30	s.	s.	
31	n.s.	-	n.s.
32	n.s.	s.	n.s.
33	n.s.	n.s.	n.s.

* due to print-out failure (see Table III-2)

n.s. = not significant

s. = significant

TABLE III-8a

Results of χ^2 Determination of Sources of Difference between Circle Sizes

Method of Blackett & Parry

Comparison	Composite Number	Organelles included	χ^2 Significance (p = 0.05)
0 nm circle to 100 nm circle	1	1, 2, 4	n.s.
	2	1 - 3	n.s.
	10	1 - 3	s.
	10	2, 4, 5	n.s.
	12	1 - 4	n.s.
	30	1 - 3	s.
	30	1, 4, 5	n.s.
0 nm circle to 230 nm circle	1	1 - 3	n.s.
	2	1 - 3	n.s.
	11	1 - 3	n.s.
	30	1 - 4	s.
	30	1, 3, 5	n.s.
	32	1 - 4	n.s.
100 nm circle to 230 nm circle	2	1 - 3	s.
	2	1, 2, 4	n.s.
	10	1 - 3	s.
	10	1, 2, 3, 5	s.
	10	2 - 5	n.s.
	11	1 - 3	n.s.
	12	1 - 3	n.s.
	16	1 - 4	n.s.
	21	1, 2, 3, 5	n.s.
	30	1, 3, 5	n.s.
	32	1, 2, 4	n.s.

TABLE III-9

Results of χ^2 Comparisons of Data

Comparison of 100 nm and 230 nm circles

for Methods of Blackett & Parry and Nadler *p = 0.05*

Composite No.	Comparison of 100 nm circle for both methods	Comparison of 230 nm circle for both methods
1	n.s.	n.s.
2	s.	s.
3	n.s.	s.
4	n.s.	s.
5	n.s.	s.
6	n.s.	s.
7	n.s.	n.s.
8	- *	n.s.
9	n.s.	s.
10	s.	n.s.
11	n.s.	s.
12	s.	s.
13	n.s.	n.s.
14	n.s.	n.s.
15	n.s.	n.s.
16	n.s.	s.
17	n.s.	s.
18	s.	n.s.
19	n.s.	n.s.
20	n.s.	s.
21	s.	s.
22	n.s.	-
23	n.s.	s.
24	n.s.	n.s.
25	n.s.	s.
26	n.s	n.s.
27	s.	n.s.
28	-	-
29	-	n.s.
30	s.	s.
31	n.s.	-
32	s.	n.s.
33	n.s.	n.s.

* failure to print-out appropriate data (see Table III-2)

n.s. = not significant

s. = significant

TABLE III-9a

Results of χ^2 Determination of Sources of Difference between Circle Sizes

Comparison of Method of Nadler to Blackett & Parry

Comparison	Composite number	Organelles included	χ^2 Significance (p = 0.05)
100 nm circle	2	1 - 3	n.s.
	{10	1 - 3	s. }
	{10	3 - 5	n.s.}
	12	1 - 4	n.s.
	18	1, 2, 3, 5	n.s.
	21	1, 2, 3, 5	n.s.
	27	1 - 3	n.s.
	{30	1 - 3	s. }
	{30	1, 2, 4	n.s.}
	32	1, 2, 4	n.s.
230 nm circle	2	1 - 3	n.s.
	3	1 - 3	n.s.
	4	2, 3	n.s.
	5	1 - 3	n.s.
	6	1 - 3	n.s.
	9	1 - 3	n.s.
	11	1, 2, 4	n.s.
	12	1 - 3	n.s.
	16	{1, 2, 3, 5	s. }
	16	{ 1, 2, 5	n.s.}
	17	1 - 3	n.s.
	20	1 - 3	n.s.
	21	{ 1 - 3	s. }
	21	{ 2, 3, 5	s. }
	21	{ 2, 3, 4	n.s.}
	23	{ 1 - 3	s. }
	23	{ 2 - 5	s. }
	23	{ 2, 3, 5	n.s.}
	23	{ 2, 4, 5	n.s }
	25	1 - 3	n.s.
	30	1, 3, 5	n.s.

TABLE III-10

Results of χ^2 Comparisons of Data

Comparison of 100 nm Circle with Blackett & Parry Method

to 230 nm Circle of Nadler Method

and 230 nm Circle of Blackett & Parry to

100 nm Circle of Nadler

Composite No.	Blackett & Parry 100 nm to Nadler 230 nm	B.&P. 100 nm to Nadler 230 nm organelles 1-3 only	Blackett & Parry 230 nm to Nadler 100 nm	B.&P. 230 to Nadler 100 organelles 1-3 onl
1	s.	s.	s.	s.
2	s.	s.	s.	n.s.
3	n.s.	n.s.	n.s.	n.s.
4	s.	s.	n.s.	n.s.
5	s.	n.s.	s.	n.s.
6	n.s.	n.s.	n.s.	n.s.
7	n.s.	n.s.	n.s.	n.s.
8	-	-	n.s.	n.s.
9	s.	n.s.	s.	n.s.
10	s.	s.	n.s.	n.s.
11	n.s.	n.s.	s.	s.
12	s.	n.s.	n.s.	n.s.
13	n.s.	n.s.	n.s.	n.s.
14	n.s.	n.s.	n.s.	n.s.
15	n.s.	n.s.	n.s.	n.s.
16	s.	n.s.	n.s.	n.s.
17	n.s.	n.s.	n.s.	n.s.
18	s.	s.	n.s.	n.s.
19	n.s.	n.s.	n.s.	n.s.
20	n.s.	n.s.	n.s.	n.s.
21	s.	n.s.	n.s.	n.s.
22	s.	n.s.	-	-
23	s.	s.	n.s.	n.s.
24	s.	n.s.	n.s.	n.s.
25	s.	n.s.	n.s.	n.s.
26	n.s.	n.s.	n.s.	n.s.
27	s.	n.s.	n.s.	n.s.
28	-	-	-	-
29	-	-	n.s.	n.s.
30	n.s.	n.s.	s.	s.
31	n.s.	n.s.	-	-
32	s.	n.s.	n.s.	n.s.
33	n.s.	n.s.	n.s.	n.s.

TABLE III-10a

Results of χ^2 Determination of Sources of Difference between Circle sizes

Cross comparison of 100 nm circle of each method to 230 nm circle of the other

Comparison	Composite Number	Organelles included	χ^2 Significance
Blackett & Parry 100 nm to Nadler 230 nm	1	2 - 4	n.s.
	1	2 - 5	n.s.
	2	1 - 3	s
	2	2, 3	n.s.
	4	2 - 4	n.s.
	4	2 - 5	n.s.
	10	2 - 5	n.s.
	18	1 - 4	s
	18	1, 2, 3, 5	n.s.
	18	2, 3, 5	n.s.
	23	1, 2, 3, 5	s
	23	2, 3, 5	n.s.
Blackett & Parry 230 nm to Nadler 100 nm	1	2 - 5	n.s.
	1	2, 4, 5	n.s.
	11	1, 2, 4, 5	n.s.
	30	1, 3, 5	n.s.

TABLE III-11

Summary of Results by χ^2 Determination of sources of Difference between Circle Sizes and Methods (from Tables 6-10a).

Method	*Composite Number*	**Organelle(s) Responsible for Difference*
Nadler 100 nm to 230 nm	25	4
Nadler 0 nm to 100 nm	27	4
Nadler 0 nm to 230 nm	1, 2, 3, 4, 7, 9, 10, 12, 14, 16, 22, 23, 25, 26, 27, 29, 31, 32	4, 5
Blackett & Parry 0 nm to 100 nm (B&P)	1	3, 5
	2	4, 5
	10	1, 4
	12	5
	30	2, 3
B.& P. 0 nm to 230 nm	1, 2, 11	4, 5
	30	2, 4
	32	5
B.& P. 100 nm to 230 nm	3, 32	3, 5
	11, 12	4, 5
	10, 21	4
	16	5
	30	2, 4

(continued)

TABLE III-11 (continued)

Summary of Results by χ^2 Determination of Sources of Differences between Circle Sizes and Methods (from Tables 6-10a)

Method	*Composite Number*	**Organelle(s) Responsible for Difference*
Nadler - B. & P. 100 nm	2, 27	4, 5
	30, 32	3, 5
	18, 21	4
	10	1, 2
	12	5
Nadler - B. & P. 230 nm	2, 3, 5, 6, 9, 12, 17, 20, 25	4, 5
	4	1, 4, 5
	11	3, 5
	16	3
	21, 23	1
	30	2, 4
B. & P. 100 nm to Nadler 230 nm	1, 4, 10, 23	1
	2	1, 4, 5
	18	4
B. & P. 230 nm to Nadler 100 nm	1	1
	11	3
	30	2, 4

* Order of organelles: 1 - rER
2 - Cytoplasm
3 - Nucleus
4 - Mitochondria
5 - Golgi

TABLE III-12

Average Relative Content of Label in Organelles from all Composites

Organelles	By Blackett & Parry Method		By Nadler Method	
	100 nm	230 nm (radius)	100 nm	230 nm (radius)
rER	12.66	12.29	9.43	7.13
Cytoplasm	68.60	67.84	76.30	82.43
Nucleus	11.49	12.70	10.33	9.08
Mitochondria	6.17	5.67	2.78	0.80
Golgi	3.48	2.31	1.15	0.40

Average Relative Areas of Organelles from all Composites

	By Blackett & Parry Analysis	By Point-hit Analysis
rER	11.15	11.27
Cytoplasm	66.60	67.88
Nucleus	11.71	11.49
Mitochondria	9.47	7.09
Golgi	2.47	2.27

TABLE III-12a

Results of χ^2 comparisons of Average Content of all composites as derived by methods of Nadler and Blackett & Parry for 100 nm and 230 nm circles

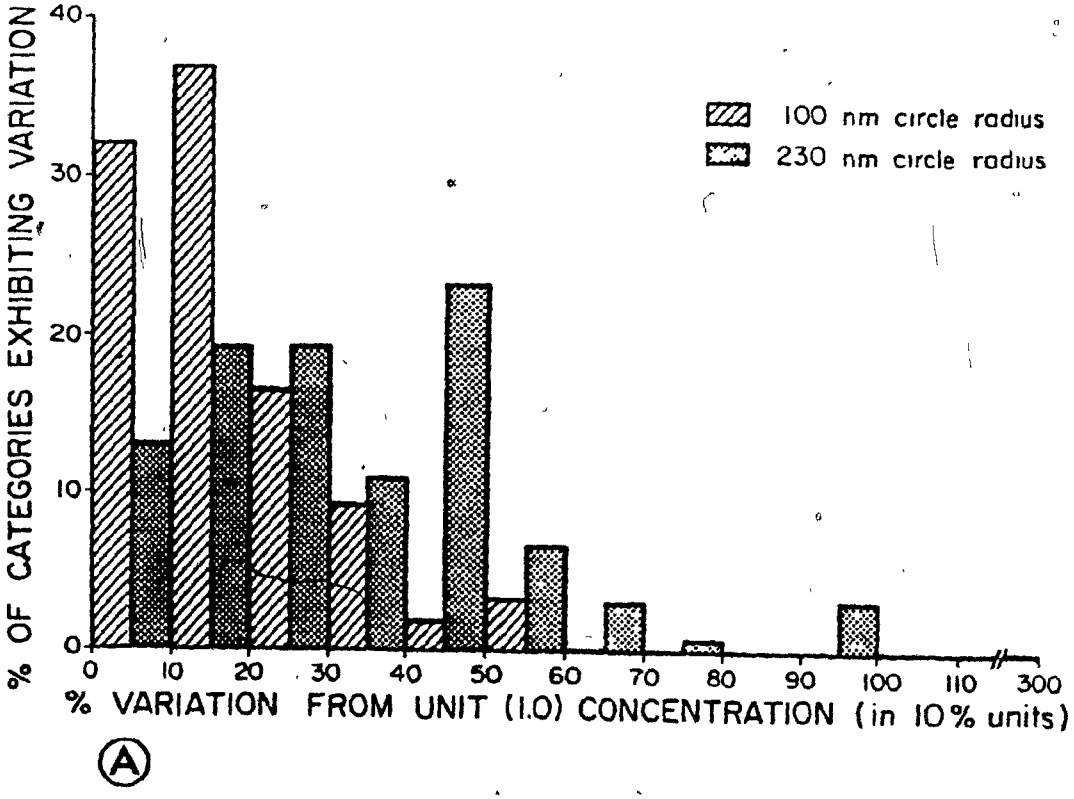
Comparison	χ^2 Significance (p = 0.05)
Blackett & Parry 100 nm circle to relative area	n.s.
Blackett & Parry 230 nm circle to relative Area	n.s.
Blackett & Parry 100 nm to 0 nm circle	n.s.
Blackett & Parry 230 nm to 0 nm circle	n.s.
Blackett & Parry 100 nm to 230 nm circle	n.s.
Nadler 100 nm to 0 nm circle	n.s.
Nadler 230 nm to 0 nm circle	n.s.
Nadler 100 nm to 230 nm circle	n.s.
Blackett & Parry 100 nm to Nadler 100 nm	n.s.
Blackett & Parry 230 nm to Nadler 230 nm	n.s.
Blackett & Parry 100 nm to Nadler 230 nm	s.
(As above but for organelles 1-3 only)	n.s.
Blackett & Parry 230 nm to Nadler 100 nm	n.s.

FIGURE LEGENDS - PART III

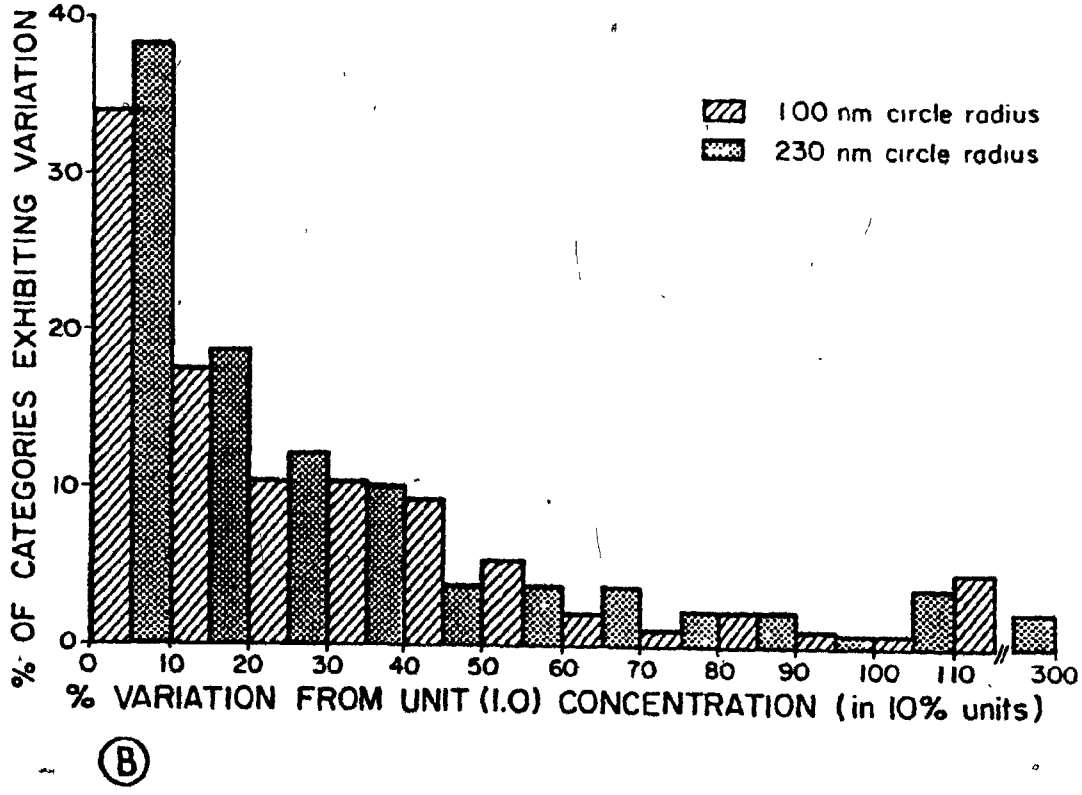
Figures III-A & B These histograms represent the percentage of variation from an assumed unit (1.0) concentration of radioactivity in hypothetical tissue standards. Results were derived by the methods of Nadler and of Blackett & Parry. Percentage variation (abcissa) is shown for both a 100 nm and 230 nm radius resolution boundary circle. The ordinate corresponds to the percentage of all categories, that is, individual classes such as rER, nucleus, etc., from individual composites 1 to 33, which exhibited variation from the assumed unit value.

Figure III-A This graph demonstrates these results after quantitative analysis by the method of Nadler. The small circle tended to show less variation than the 230 nm circle but the majority of results from both circle sizes exhibited less than 50% variation from unit concentration.

Figure III-B This graph shows the results derived after analysis by the method of Blackett & Parry. Both circle sizes showed essentially equivalent variation (i.e., no significant difference by χ^2, $p = 0.05$). About 10% of all categories were found to vary by greater than 50% from unit concentration, while about half of these (5% of total) varied by greater than 100% and up to 300% from assumed unit concentration values.


% OF CATEGORIES EXHIBITING VARIATION
40
30
20
10
0
100 nm circle radius
230 nm circle radius
0 10 20 30 40 50 60 70 80 90 100 110 300
% VARIATION FROM UNIT (1.0) CONCENTRATION (in 10% units)

(A)


% OF CATEGORIES EXHIBITING VARIATION
40
30
20
10
0
100 nm circle radius
230 nm circle radius
0 10 20 30 40 50 60 70 80 90 100 110 300
% VARIATION FROM UNIT (1.0) CONCENTRATION (in 10% units)

(B)

Figure III-C. This is a sample of the print-out obtained with the Hewlett-Packard (9810A) calculator for the method of Nadler, as similarly shown in Part II. The data is for Composite 6 (Fig. III-6) and was derived using a 100 nm resolution boundary circle radius. Note the increased counts attributed to the cytoplasm after the analysis output.

Input

Exclusive Grains

rER	3.00+
Cytoplasm	1.00-
Nucleus	30.00+
Mitochondria	3.00+
Golgi	1.00+

Shared Grains

R+C	4.00+
R+M	0.00+
C+N	7.00+
C+M	5.00-
C+G	1.00+
M+G	0.00+
R+C+M	0.00+
C+M+G	0.00+

Volumes

R Vp	9.00+
R Vo	11.10+
C Vp	49.00+
C Vo	48.90+
N Vp	35.00+
N Vo	28.90+
M Vp	6.00+
M Vo	8.90+
G Vp	1.00+
G Vo	2.10+

Output

T_{EM}	100.00
Loop Number	3.00+
Sensitivity(p)	0.05
Iterations	513.00

TRUE COUNTS

rER	6.45
Cytoplasm	54.39
Nucleus	32.96
Mitochondria	3.18
Golgi	1.02
± S.D.	0.01

Computation Time

Hours	0.00
Minutes	5.00
Seconds	16.00

End Print

Figure III-D. This is a sample of the computer print-out after analysis by the method of Blackett & Parry. The data shown is for Composite 2 (Fig. III-2) derived with a resolution boundary circle of 100 nm radius. The distribution of hypothetical grains ("Original Grains") is compared to that of the real grains whose distribution is based on the assumption of uniform incorporation of isotope. The goodness-of-fit is then determined by χ^2. The computer program predicts the estimated *true* distribution of activity and, in addition, the relative areas of organelles.

NKN	SLK	IPRINT	ICØN	MAXIT	ESCALE	E
30	3	0	1	50	50	•100 NUG

-1•32 •86594E+00 -•28691E-02 1•20
2•36

ØRIGINAL GRAINS

SITE	R	C	N	M	G	R+C	N+C	M+C	G+C
rER	400	4	100	4	4	200	4	4	4
Cytoplasm	100	4700	4	200	4	300	300	300	4
Nucleus	4	4	4	4	4	4	300	4	4
Mitochondria	4	300	4	100	4	100	4	200	4
Golgi	4	300	4	4	4	4	4	4	100
REAL GRAINS	100	680	20	20	4	30	30	90	30
HYP• GRAINS	•1	645	19	39	3	84	35	66	31
CHI SQD•	4•5	1•9	•0	9•6	0•	34•5	•7	8•5	•0

TØTAL CHI SQUARED 59•6 WITH 3 DEGREES ØF FREEDØM

CØMPUTED FIT WITH ESTIMATED STD• ERRØRS

SØURCE	GRAINS/GRID PØINT	REL• ACTIVITY	REL• AREA
rER	•171 ± •021	12•3 ± 1•4	8•9 ± •3
Cytoplasm	•100 ± •007	63•6 ± 3•9	72•0 ± •4
Nucleus	•000 ± •005	•0 ± •2	4•1 ± •2
Mitochondria	•160 ± •038	11•5 ± 2•6	8•9 ± •3
Golgi	•294 ± •052	12•5 ± 1•9	5•3 ± •2

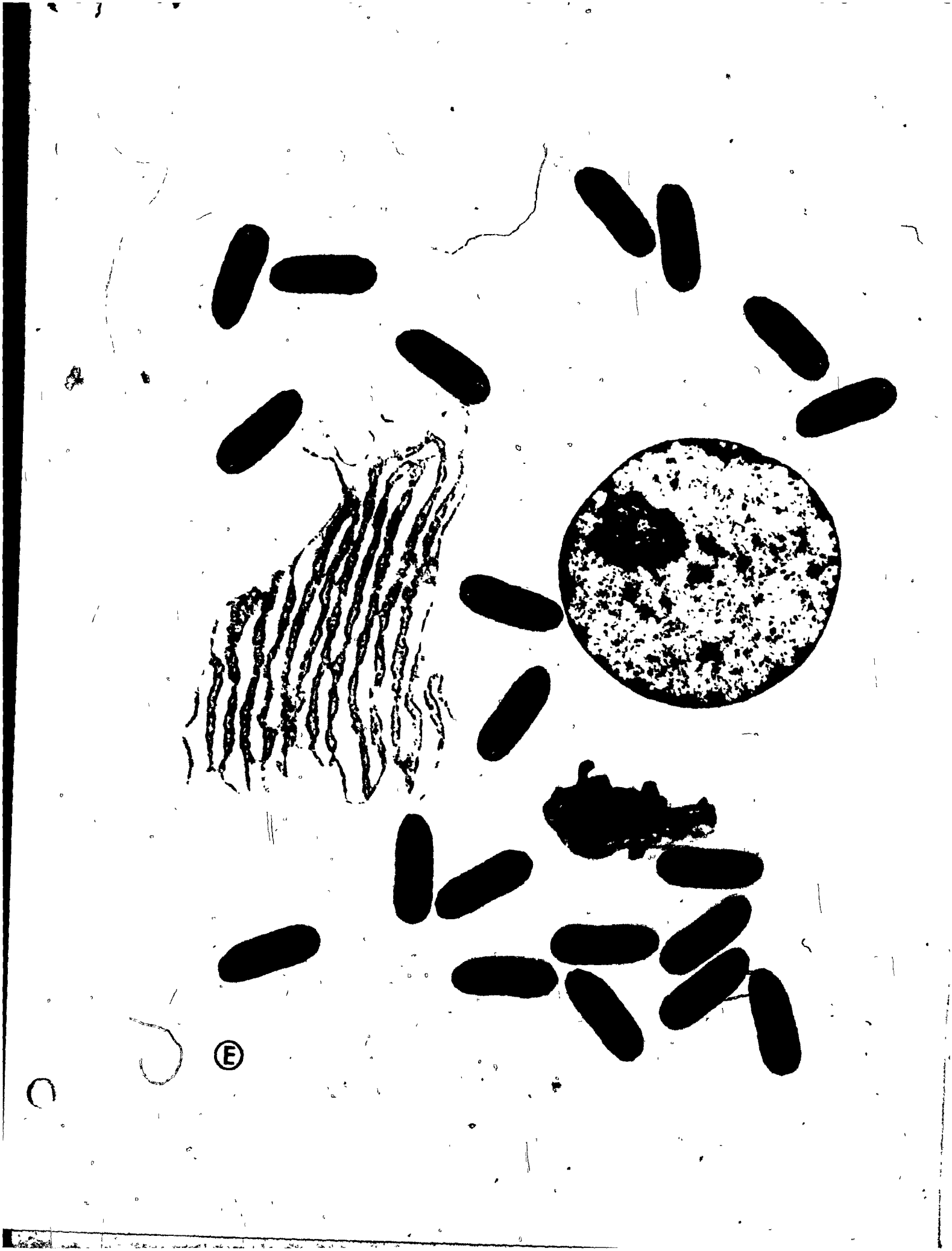
STD• ERRØR ØF CHI SQD• 13•5

Ⓓ

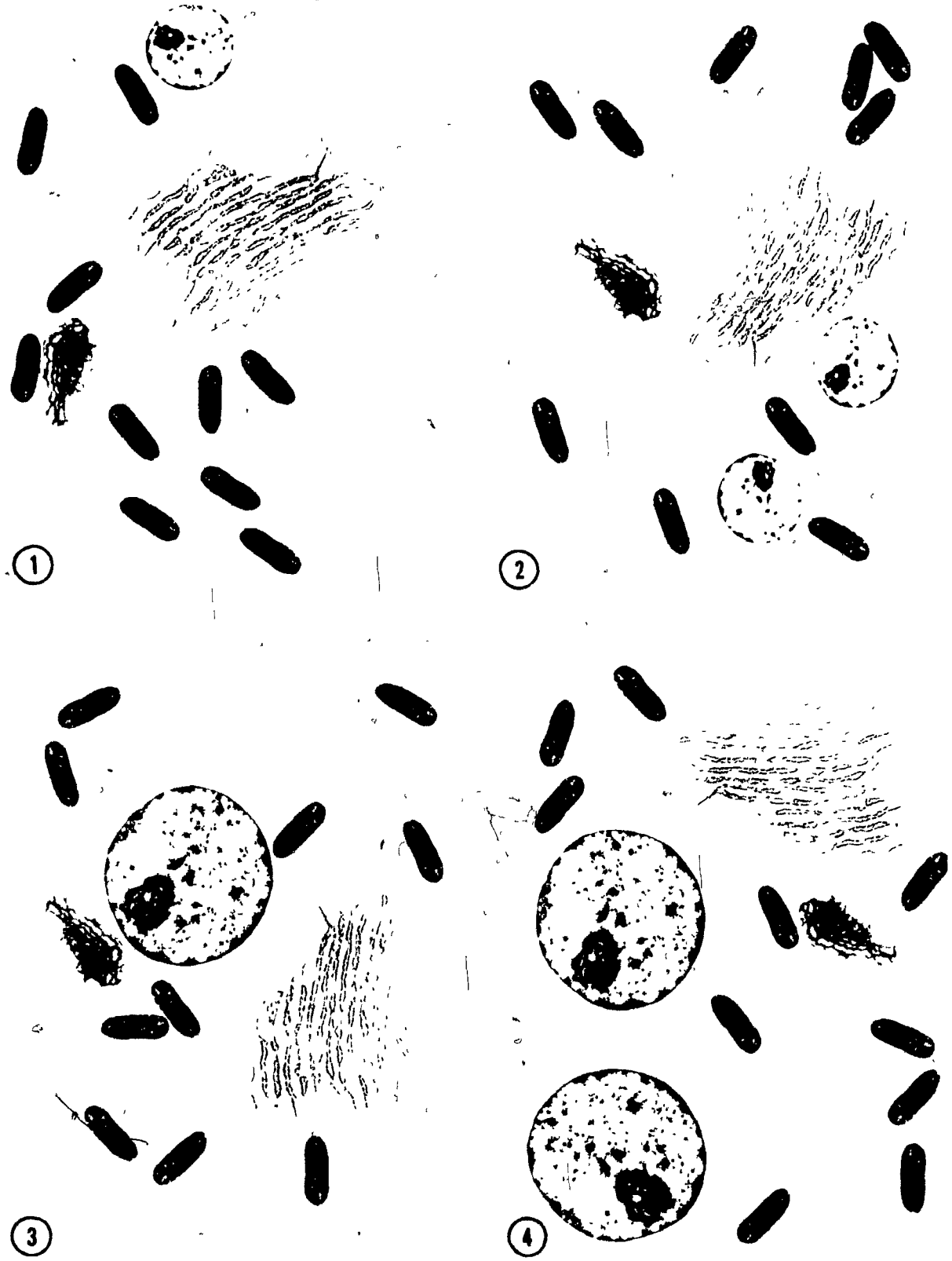
Figure Legends - Part III

Figure III-E. This is a sample of the hypothetical tissue standard composite photographs used in these studies (composite No. 22). Magnification of the nucleus is equivalent to 10,000X. Magnification of other organelles equivalent to 25,000 X. Reduced to 3/4 original size.

The use of hypothetical cell models allows ultrastructural variables to be controlled and simplifies grain counting. This provides a system in which quantitative methods can be compared under many possible conditions.



Figures III-1 to III-33. These figures correspond to hypothetical tissue standard composite photographs 1 to 33. For ratios of organelle size and quantity, consult Tables IIIA and B (Materials and Methods). Original sizes reduced for thesis presentation. Magnification equivalent to approximately 4,000 X.


1
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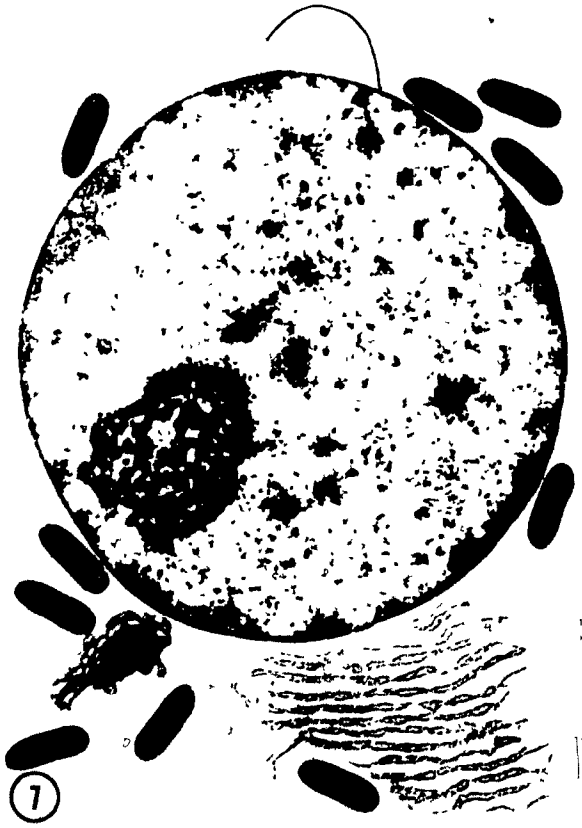
3
4

5

6



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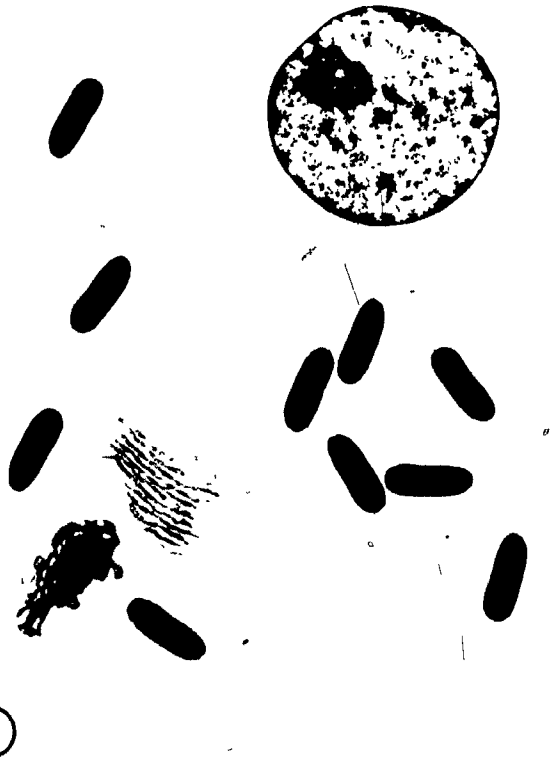

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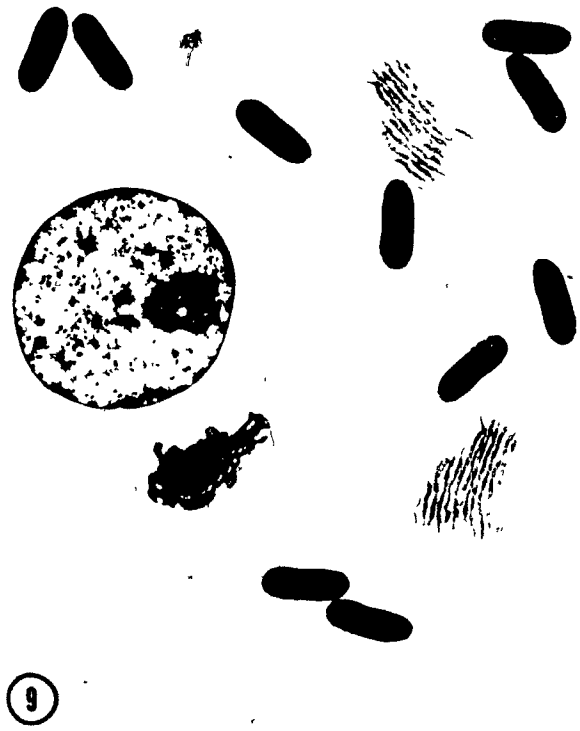

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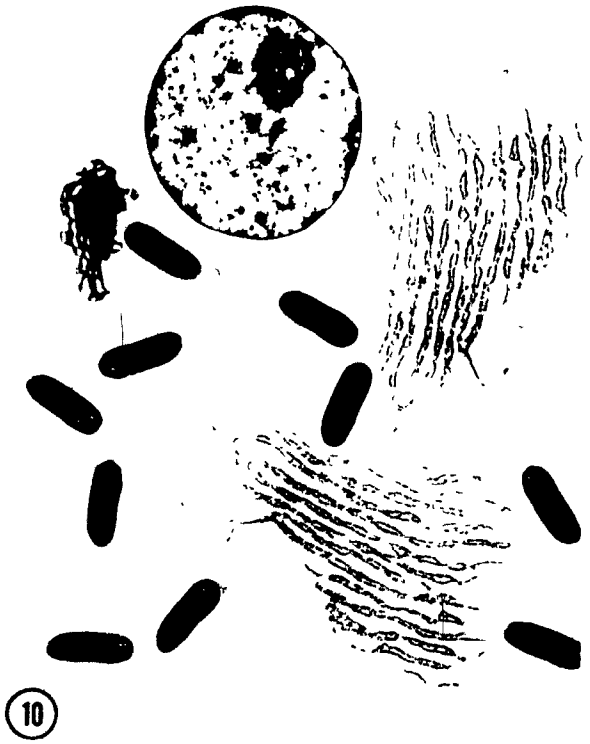

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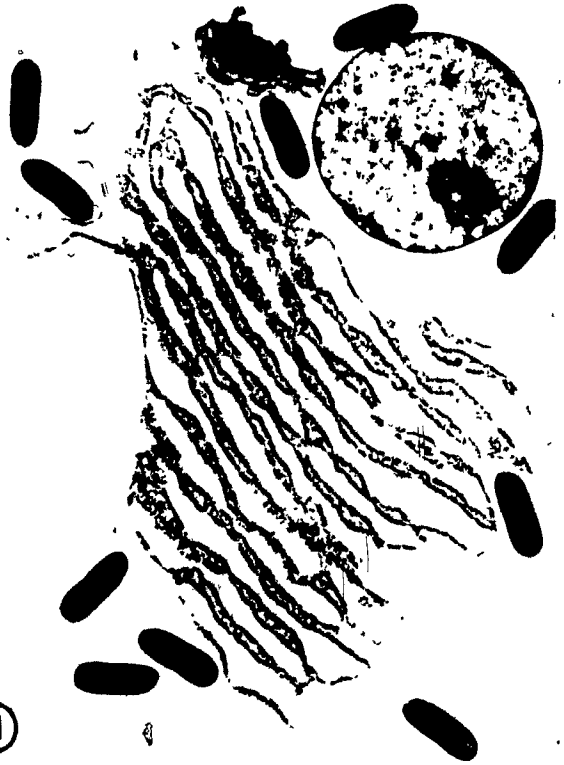

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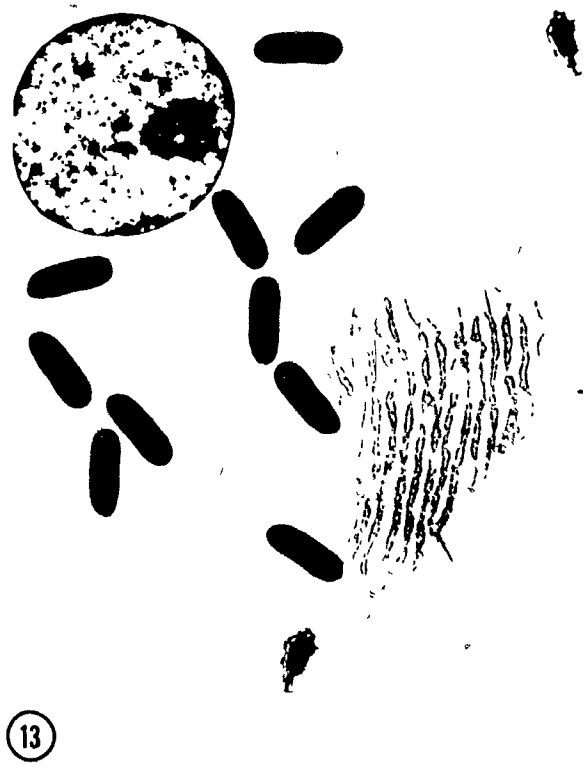

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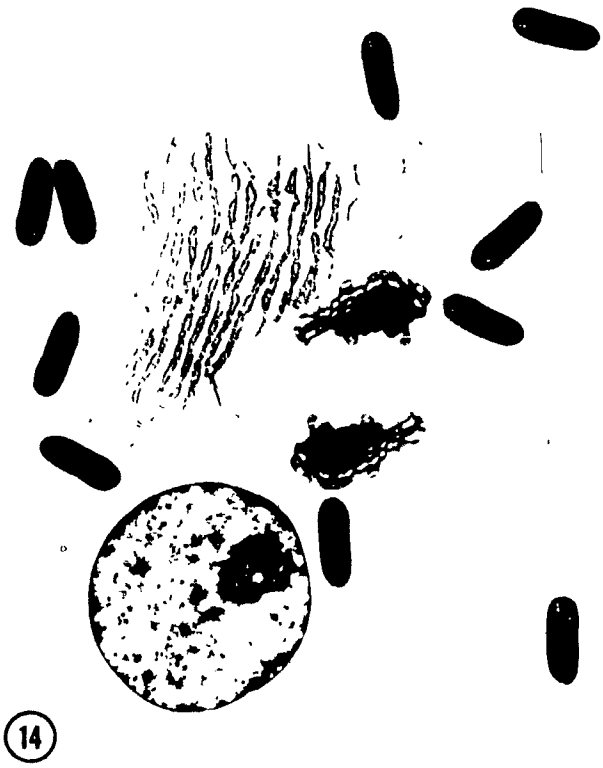

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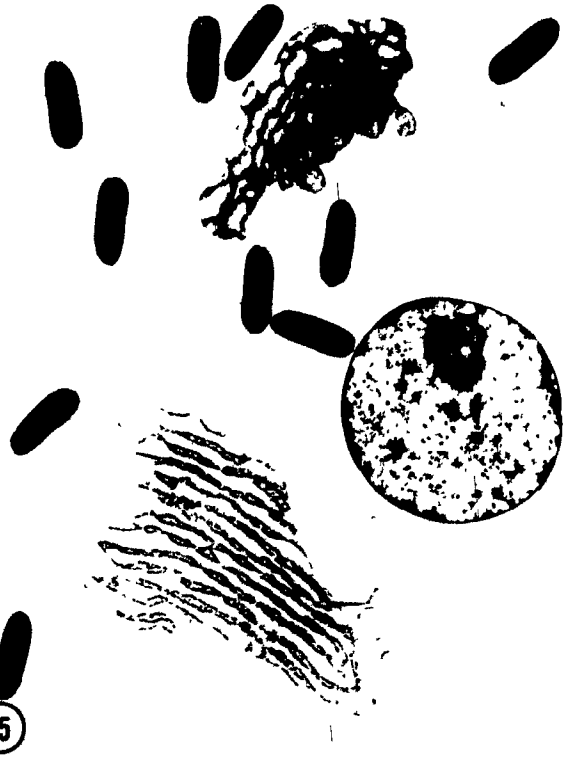

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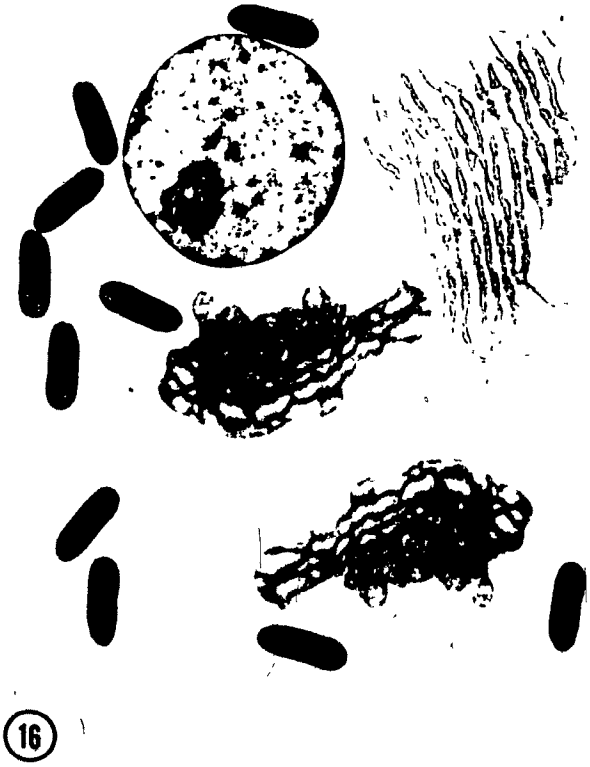

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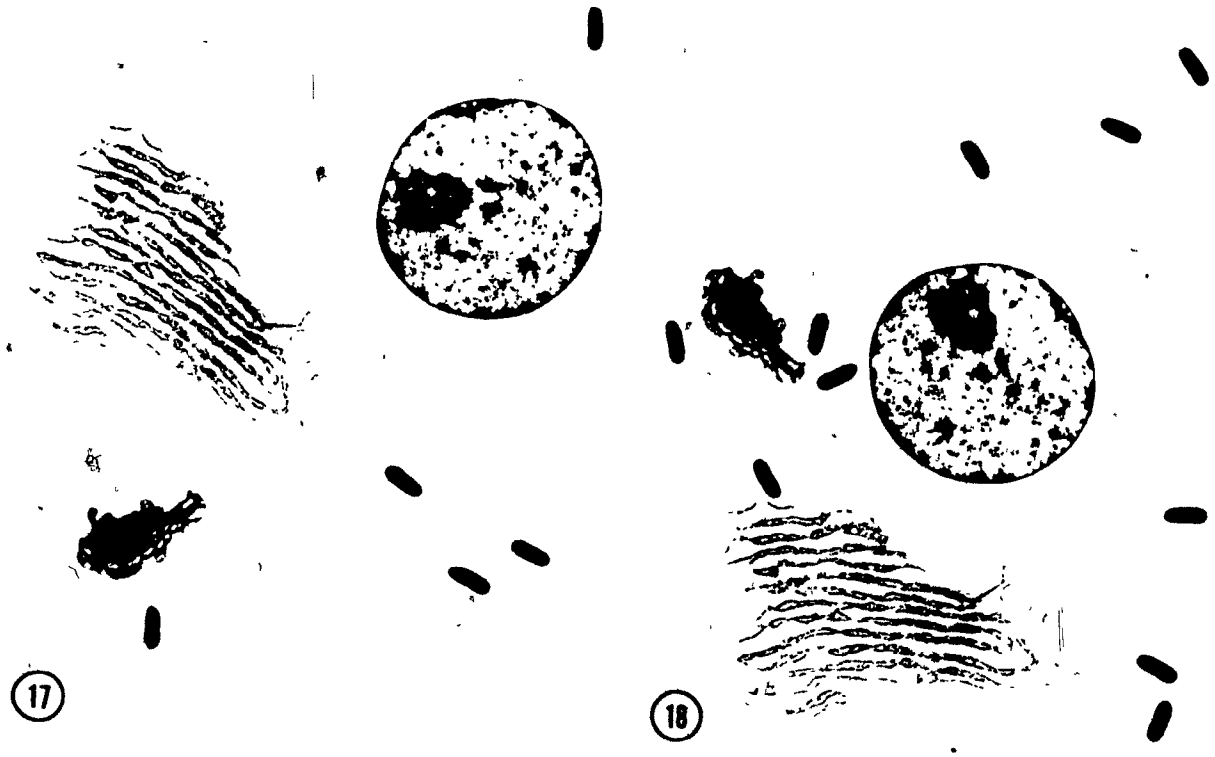

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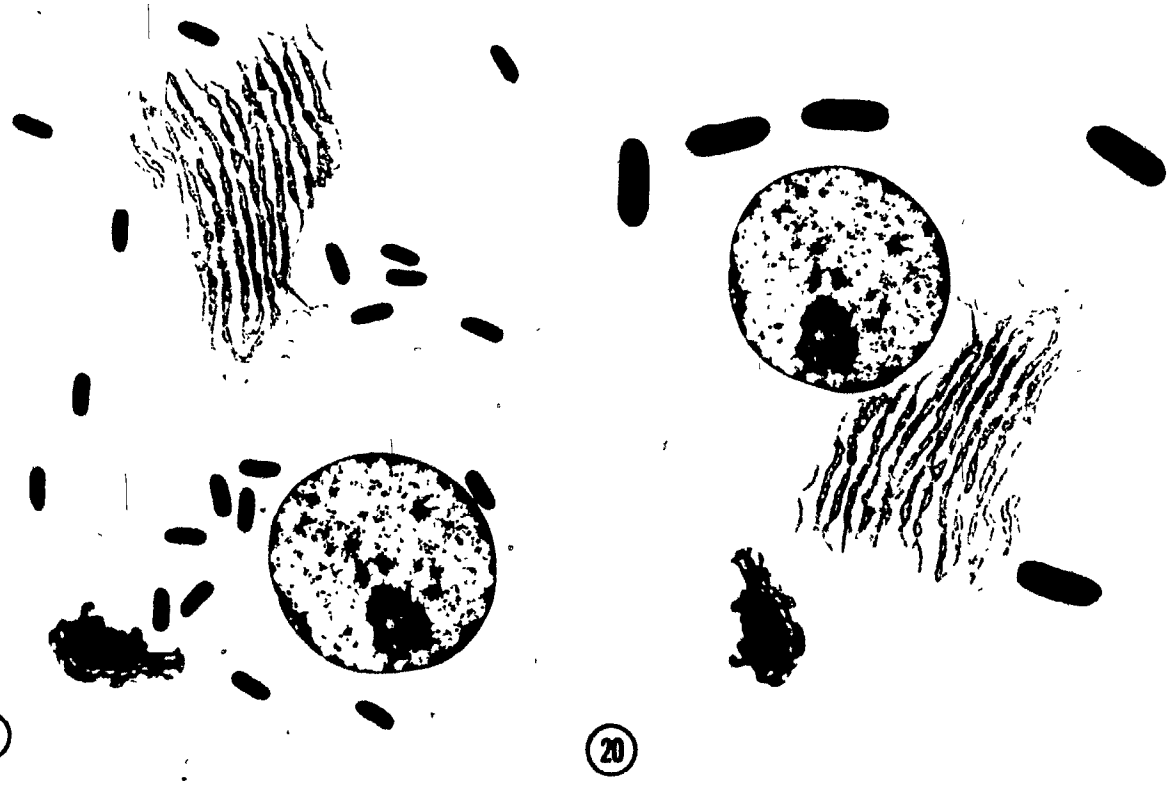

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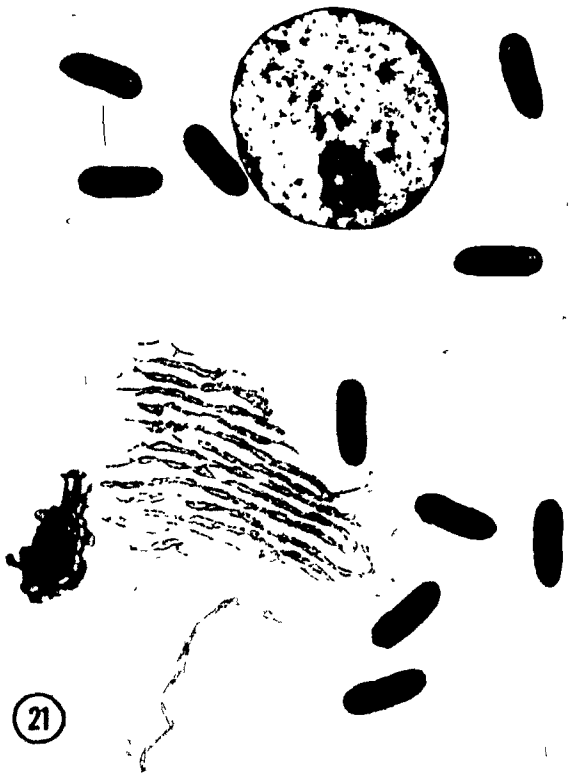

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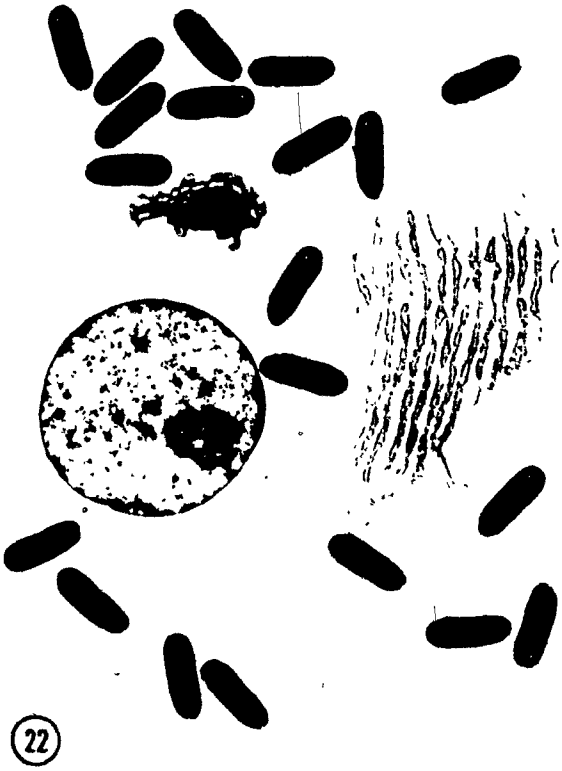

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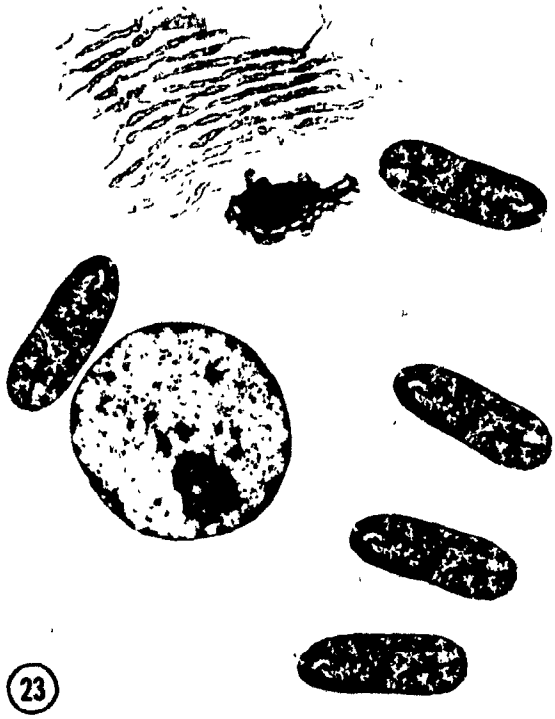

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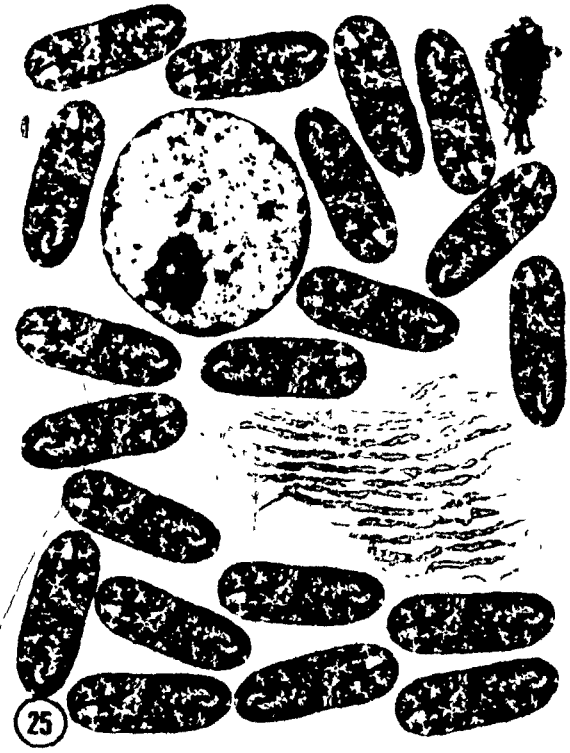
(24)



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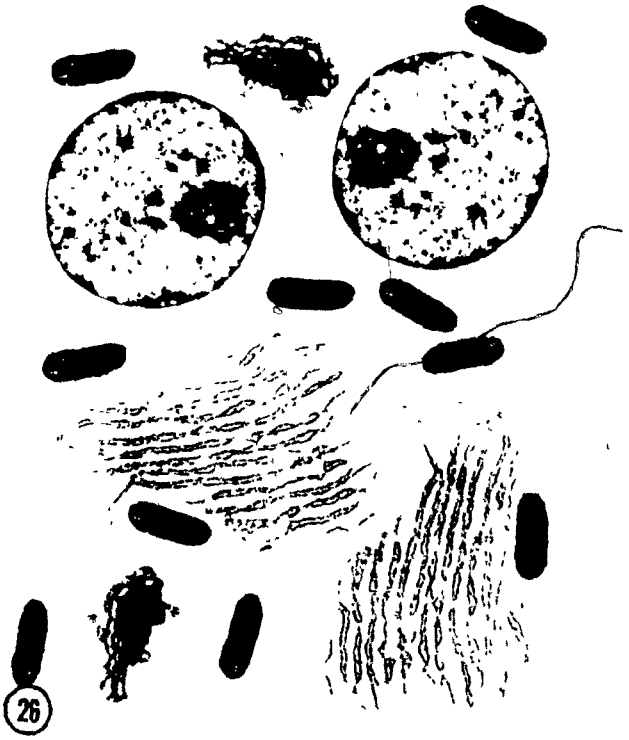

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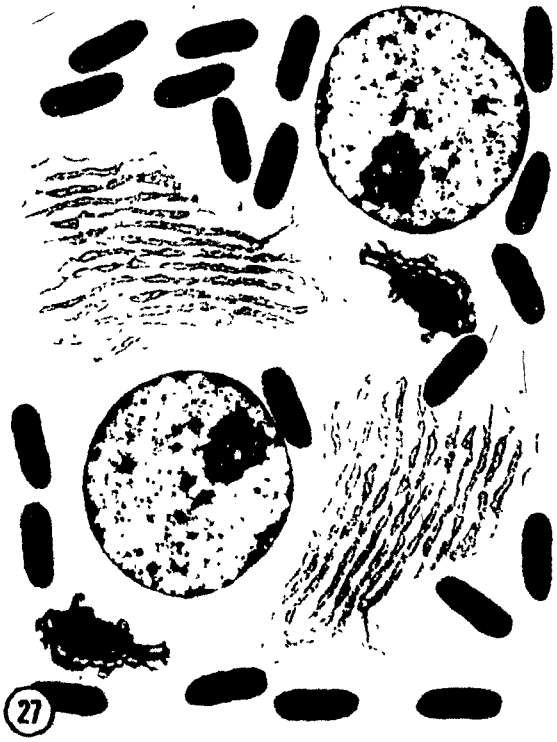

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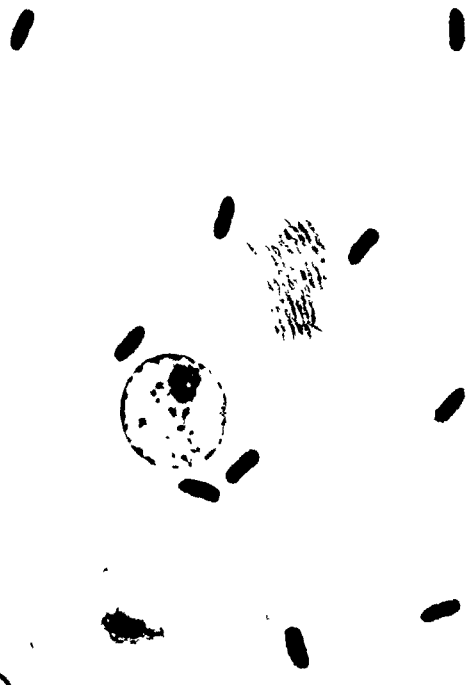

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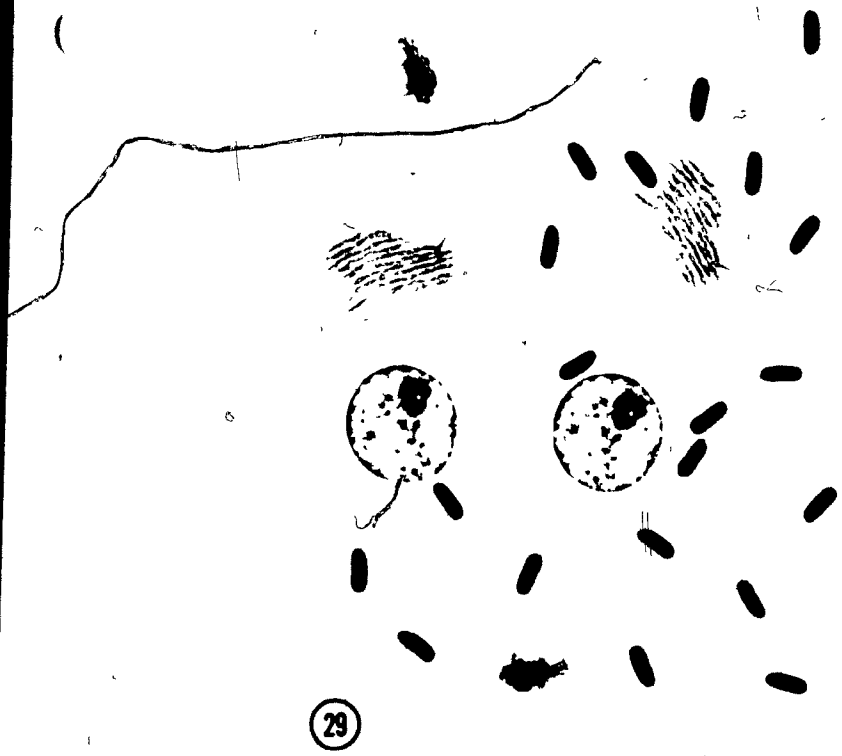

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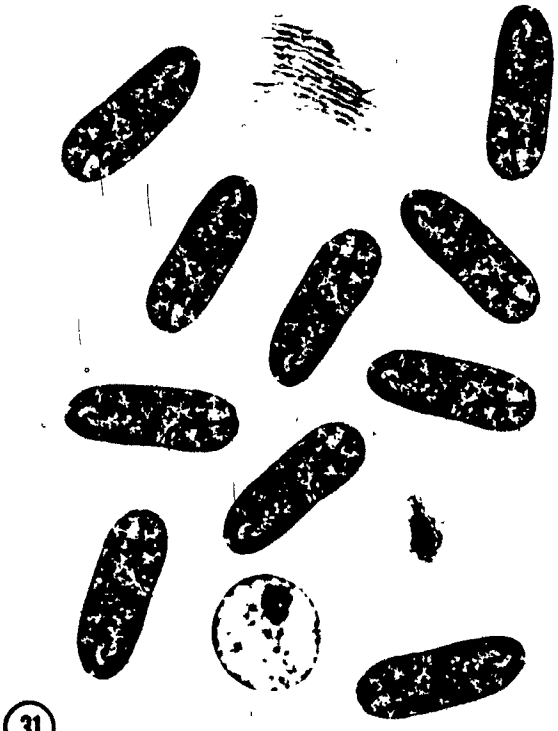

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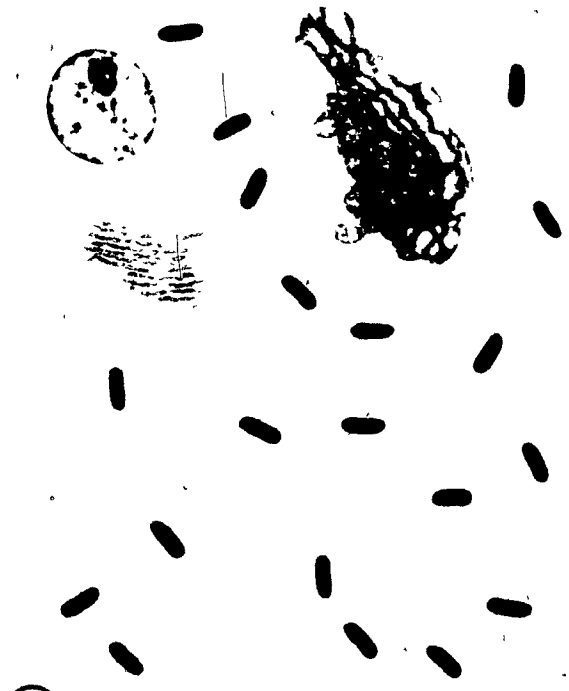

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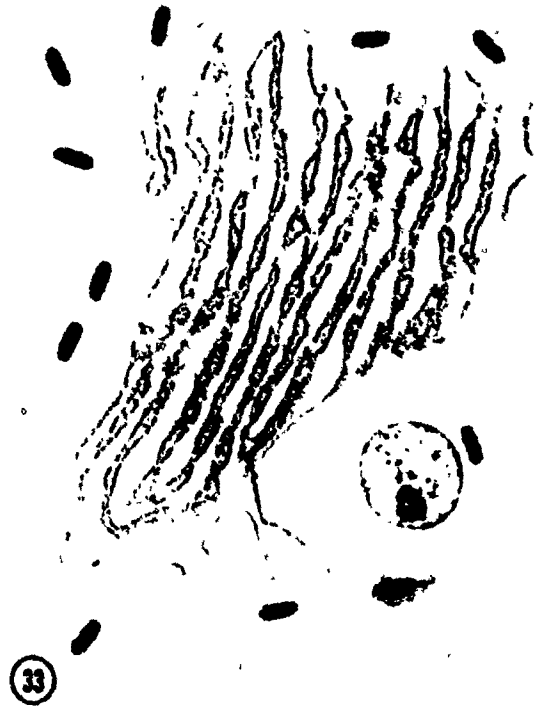

(33)

DISCUSSION

The number of developed silver grains observed in a radioautograph being proportional to the amount of incident radiation caused by nuclear disintegrations, provides a rational basis to quantify the radioautographic reaction. The two main techniques which are in current use for the quantitative evaluation of radioautographs, that of Nadler and of Blackett & Parry, are both subject to two requirements. First, it is necessary to draw a resolution boundary circle around each grain in order to decide which structures in the electron micrograph probably contain the radioactive label responsible for having caused that grain to form. Secondly, some method must be applied to determine what proportion of grains which are associated with more than one structure, when enclosed by the resolution boundary circle, should be assigned to each of the structures involved.

This thesis attempts to answer the following two questions:

1) Does the size of the resolution boundary circle matter?
2) Of the two methods in question for the analysis of shared grains, which is better suited for what purpose, and what is the order of accuracy?

It is hoped that the principal contribution of this work will have served to simplify, if possible, the rather complex methodology of analysis which has been heretofore necessary to use. In addition, this investigation may provide the order of accuracy with which it is possible to discriminate relative content of label between organelles.

Line Source Radioautography

The technique of Salpeter to measure the resolution of the radio-

autographic method has been applied to decide whether a particular structure seen in an electron microscope radioautograph is labelled or not (e.g., Budd & Salpeter, 1969). This is done by comparing the expected grain distribution around a prepared radioactive line source to the observed distribution of grains around specific assumed sources in radioautographs. One of the features introduced by this method was the concept of the half-distance value (HD), which is the distance from the radioactive source within which half of all developed grains are found. This value is thus equivalent to a 50% probability that the source of radioactivity responsible for that grain will be within the distance from the centre of that grain corresponding to that probability. The HD also acts as a measure of the resolution of the radioautographic technique under various experimental conditions.

During the course of present investigations, the effects of several parameters on the HD value have been examined to assess their relationship to the shape, size and distribution of grains seen in electron microscope radioautographs. Since HD is a measure of resolution, the less the value is, the better will be the resolving power.

Validity of the Line Source Resolution Specimen

In establishing the HD values and the pattern of grain distributions around radioactive line sources, certain errors are involved. These should be accounted for when applying this information to radioautographs of experimental tissue sections. There are also factors caused by the statistical implications of grain counting, such as the effects of the cut-off distance and the quantity of grains included, which may affect either or both the expected (line source) and observed (tissue) HD values.

Sources of Error

Due to the statistical fluctuations of grain counts in these analyses, since less than 1,000 grains were counted for each study, a margin of at least ±10 nm should be assumed for values determined from histograms of experimental grain density distributions around line sources (Salpeter *et al.*, 1969). Furthermore, since grain measurements are made from the centre of a 50 nm thick line source (as shown in Fig. I-1), a possible deviation of up to ±25 nm is introduced for values of distances from grains to the line. Because of the fact that a particular radioactive source could be on either edge of the line (a line source is a linear collection of discrete point sources), or anywhere across its thickness rather than exactly in the middle, this should therefore be accounted for. In the system of measurement used for these studies, an additional error of ±2 nm, although a negligible amount, should be included to account for reading of distances with the aid of a calibrated optical reticle (as described in Materials and Methods).

Thus known possible errors which can be distinguished statistically may involve up to at least ±37 nm for values derived by measuring grain density distributions around known radioactive line sources.

There are also possibilities of introducing errors which cannot be easily calculated. Establishing the centre of the developed silver grain presents an obstacle which may create unaccountable problems in the determination of grain distributions. Further complications may result when applying the information derived from line source studies to grain distributions observed in radioautographs of experimental tissues. Similar errors to those involved in interpreting the line source specimen would likely be

introduced. In fact, the possibility of doing so is actually greater, because measurements are made to the nearest assumed radioactive source. In histological sections, this source may not necessarily be in the form of a line, but could include many shapes and sizes of organelles. Conversion of resolution values for a line source to those for a point source (Bachmann & Salpeter, 1965; Salpeter *et al.*, 1969, 1977) or to those for a hollow or solid disc, band or circular source (Salpeter *et al.*, 1969), involves certain mathematical manipulations and again possible introduction of errors.

Effects of Cut-off Distance

Reducing the cut-off distance resulted in a decrease in the definitive HD value. However, the decrease was not as great as might have been expected by cutting off counts at 3 HD instead of 10 HD (Table I-4). This reduction accounted for a decrease in HD of about 20 to 30 nm. Thus, as suggested by Salpeter *et al.* (1969), a cut-off distance of 10 HD does not exclude a significant number of grains, while cutting off too close to the radioactive source will have an obvious effect on the HD value, apparently improving the resolution. In actual fact, however, by omitting grains at further distances from the line source, a situation is created where the HD is not representative of the real grain distribution. Accounting for statistical error though, if a margin of as little as ±10 nm is assumed for all HD values (Salpeter *et al.*, 1969; Salpeter & Salpeter, 1971), then the difference between a cut-off distance of 10 HD units and 3 HD units is interpreted as a minimum difference of only 10 nm, which may be a negligible amount. In addition, this speculation is dependent on the shape of the curve of individual grain distribution patterns, and especially on the

extent of the "tail" of the curve. For the study in question here, with ^{3}H, Ilford L4 emulsion and D19b developer, the actual number of grains from the 677 included at 10 HD, which were excluded by cutting off at 3 HD, was only a sum of 80 grains, or about 12% of the total. This may explain why the difference created by cutting off at 3 HD was not as great as might have been anticipated, and at the same time indicates that a cut-off distance could be decreased to as low as 2 HD units without having a major effect on the definitive HD value, depending on the shape of the distribution curve. Thus a resolution value of 58 nm reported by Miura & Mizuhira (1965) may not be as far from those suggested by other investigators, if this discrepancy is taken into account.

Effects of Quantity of Grain Counts

On a statistical basis, the error involved in counting grains is proportional to the square root of the number of grains included in the counts. Therefore, the more grains that are counted, the less the proportionate error becomes. From these studies on the Ilford L4 emulsion with ^{3}H developed by D19b, based on experimental observations of actual grain distributions (Table I-3), a quantity of only 50 grains was shown to reduce the HD value considerably, and the interpretation is that this value of grains was too low and must be increased. From the NTE study, separate observations indicated that a total of 137 grains might have been low as well (Tables I-1 and T-2). Speculation as to apparent differences in this preliminary investigation supports the notion that, again, too few grains were included.

From both the ^{125}I study with the GSPD (data not shown) and ^{3}H with the D19b developer (Table I-3), it was observed that counting approximately 250 to 350 grains resulted in essentially the same HD value as that found

by counting 600 to 700 grains. Thus, from practical data rather than statistical theory, it can be suggested that counting much less than 150 grains will create unwanted fluctuations in the determination of resolution values. At the same time, counting more than 300 or so grains does not seem necessary, since beyond this value even doubling the number of grains to 600 does not affect the final HD value derived. It must be kept in mind though, that decreasing the quantity of grain counts unavoidably increases the comparative standard error and thus the more grains included, the less the variation in measurements will become.

Advantages of using a Line Source Resolution Specimen

Although it is technically difficult to produce a line source profile section (See Materials and Methods), and in spite of other apparent drawbacks, positive aspects of this method cannot be overlooked. The method adheres strictly to the laws of probability and source-detector geometry. It is based on the foundations of systematic experimental observation and provides an adequate reflection of the resolution of radioautographic preparations. Under certain conditions, where *quantitation of the relative amounts of radioactivity is not required* (e.g., Fertuck & Salpeter, 1974) and where the *location of a unique radioactive source in experimental tissues is suspected* and must be verified (e.g., Bergeron *et al.*, 1977), then the preparation of a line source resolution specimen may be the most relevant technique to employ for the analysis of electron microscope radioautographs.

Effects of Various Parameters on Resolution

Effects of Isotope

From these investigations, the isotopes studied demonstrated differences in resolution only when using the chemical developer D19b, giving rise

to a filamentous grain (Figs. I-2 and I-4). However, when the solution physical process of development was employed (GSPD), giving rise to a fine grain (Figs. I-3 and I-5), no differences due to the energy of the isotope were observed (See Table I-1). It has previously been demonstrated that resolution with ^{125}I is expected to be better than that of ^{3}H (Fertuck & Salpeter, 1974a; Salpeter *et al.*, 1977). The developers used in those earlier tests were also of the chemical and solution physical type, resulting in various shapes and sizes of developed grains. However, the solution physical developer, paraphenelyendiamine (Caro, 1962; Caro & Van Tubergen, 1962) used in those studies produced a larger silver grain than did the GSPD used in the present study (Kopriwa, 1975). In addition, the grain produced by the Gold-EAS developer in Salpeter's work (Wisse & Tates, 1968; Salpeter & Szabo, 1972) was smaller than that produced by development with D19b (Kopriwa, 1967a).

It is believed, therefore, that when the radioautograph is developed with the solution physical developer manufactured by Agfa-Gevaert, then no differences in resolution due to the isotopes ^{125}I or ^{3}H are observed.

Possible explanations for this phenomenon are open to speculation. It is plausible that latent images that are further from the radioactive source may not be developed by the GSPD, even after gold latensification (Bachmann & Salpeter, 1964; Salpeter & Szabo, 1972). If this were true, then there could be a critical distance away from the radioactive source beyond which latent images will not be developed by this particular fine grain method. On the other hand, the filamentous developers in common use may be comparatively "over-sensitive" in developing latent images at distances from the radiation, which add to the complicated analysis of grain

distributions. In other words, it is feasible that the direct chemical development produces many grains, so distant from the source that they are of no value to the localization of that source of radioactivity in the tissue.

In any case, the silver grains produced by the solution physical development with GSPD appeared closer to the radioactive source regardless of whether the isotope was ^{125}I or ^{3}H, and might have been independent of developed grain size. However, with filamentous grain developers such as Kodak D19b, the resolution of ^{125}I is unmistakably better than that of ^{3}H.

Effects of Emulsion

In the preliminary study on the differences between the Kodak NTE (Fig. I-6) and Ilford L4 emulsions (Fig. I-2), the NTE produced a better resolving power or a lower HD value than that for Ilford L4, under similar conditions of isotope (^{3}H) and developer (D19b). The Kodak Nuclear Track Emulsion is one of extremely low sensitivity. Radioautographs exposed for 103 days produced only 137 developed silver grains from ^{3}H-labelled line sources, as opposed to shorter periods of 49 and 63 days, where six times that number of grains was produced using the Ilford L4 emulsion. In addition, the NTE has been found to provide inconsistent results and is not generally accepted as a good quality emulsion (Kopriwa, 1975; Salpeter *et al.*, 1977).

Concerning differences seen in grain distribution patterns over the Epon and celloidin sides of the line source, these might have been due to the reduced sensitivity of the NTE. That is, the low density of latent images might have been caused by absorption of radiation by Epon in such a way that the lower sensitivity emulsion could not detect the energy particles, where the Ilford L4, for example, could. Although the size of the silver

bromide crystals comprising the NTE emulsion are smaller (0.05 μm) than those of the Ilford L4 (0.14 μm), the loss of sensitivity and undesirable fluctuations in developed grain size make this emulsion impractical for accurate detection of radioactive disintegrations in experimental tissues.

Effects of Developer

As previously discussed, the GSPD gave a better resolution over that of D19b (See Table I-1). Since the geometric centre of a filamentous grain is not necessarily the site of the latent image from which the filament begins to grow upon development, then measuring the distance from the grain to its known source of radioactivity, using the grain centre as the reference point may introduce a margin of error. The work of Salpeter *et al.* (1977) showed that ^{125}I resolution is little affected by the size of the developed grain. However, there is a discrepancy between those results and the data from this present study concerning the relative sizes of the developed grains, as mentioned in relation to the effects of isotope. This possibly accounts for the smaller differences observed between chemical and solution physical developers in Salpeter's investigation as compared to the greater differences between HD values for the two types of developers used in these experiments.

Since the "classical" filamentous grains grow away from and around the latent image, even if this growth was assumed to be symmetrical, as suggested by Salpeter *et al.* (1977), it would be difficult to demonstrate that the chosen geometric centre was actually the site of the latent image. Especially where the filament produced is comparatively large, this could be involved in causing a misinterpretation of the measure of resolution.

From the evidence provided by the results of these studies, the GSPD

produced a more compact grain than found with D19b (Figs. I-2 to I-5) and thus, whether directly because of the grain size, or indirectly due to the lack of developed latent images at greater distances from the source, caused a real and significant reduction in the HD value for both isotopes ^{125}I and ^{3}H.

Effects of Absorption by Epon

The results of these tests indicated that the effects of absorption of energy particles by the embedding medium Epon (self-absorption), were negligible for a section thickness of 100 nm (See Table I-2). In an indirect manner, this is supported by the observations of Salpeter *et al.* (1977). Their data indicated no major differences between HD values for sections of 50, 100 and 150 nm thickness. The present study indicated no differences between 0 nm thick (i.e., no Epon) and 100 nm thick distances of Epon, through which many but not all of the energy particles released must pass in order to reach the emulsion.

The only exception to this observation was in regards to the NTE emulsion, where there was a difference in normalized grain distribution patterns over the Epon and celloidin sides of the line source. This could have indicated that either too few grains were counted or that there was some self-absorption and the NTE was not sensitive enough to detect the nuclear decays. However, no significant difference was observed over either side of the line when actual grain counts were considered and, since the study involving the NTE was only a preliminary analysis, further investigation should be made to determine the relative likelihood of these events.

Effects of Exposure Ti

For relatively short durations of exposure of the emulsion to radio-

active disintegrations occurring within an experimental tissue, increasing numbers of grains will be developed in a linear relationship to increasing time, by increasing the quantity of latent images produced in the silver bromide crystals (Kopriwa, 1975). The evidence of these tests support that rationale, since the length of exposure had little, if any, effect on the resolution of the technique. The HD derived from grain distributions for ^{3}H, after 49 days of exposure did not differ from that after 63 days (Table I-3).

Quantitative Electron Microscope Radioautography

A quantitative technique for electron microscope radioautography must fulfill two criteria. It must be capable of determining firstly, the location of radioactive sources, and secondly, the relative amounts of radioactive label in the ultrastructural organelles of experimental tissues.

In order to appraise the application of quantitative techniques to E.M. radioautography, the effects of the size of the resolution boundary circle on interpretations of silver grain distributions in radioautographs were examined. Since a resolution circle is required by both of the two main techniques available for this purpose, both methods were studied in relation to a small (100 nm) and large (230 nm) circle radius. These results were compared to data derived using a nil (0 nm) circle where no further statistical analysis was necessary, since there were no shared grains.

Observations were made under experimental and hypothetical conditions. For the former, electron microscope radioautographs of *experimental tissue* sections of thyroid follicles (kindly provided by Dr. S. Cassol) taken 4 hours after the injection of ^{55}Fe (See Materials and Methods - Part II) were used as a substrate for the comparisons of the methods and circle sizes.

For the latter, various *hypothetical tissue* standards or cell models were prepared from biological material with the aid of photography and computer-assisted randomization (See Materials and Methods - Part III). For these studies, uniform incorporation of a hypothetical radioactive tracer was assumed.

Comparisons were subsequently made between circle sizes and methods under these criteria.

The Size of the Resolution Boundary Circle

Experimental Tissue Observations

Under the experimental conditions described in Part II, results derived by the method of analysis of Nadler showed no significant differences between the 100 nm and 230 nm circle radii. Moreover, there was no difference between these and those derived with the nil circle whereby data was established by directly scoring the structure underlying the centre of the grain as the exclusive source of radiation (Table II-2). The greatest relative differences between circle sizes were seen in association with grain interpretations for the mitochondria, membranes and rER. In most cases, it was these structures which shared the largest proportion of silver grains (See Discussion Table 1). On theoretical grounds, by using a smaller circle, there is comparatively less chance of including possible radioactive sources further from the grain centre. This explains the differences (though negligible) observed between circle sizes for some of the data. Due to the diffuse distribution of basal and lateral membranes which actually comprise a small percentage of the total area of the cells, grains shared by this and other organelles tend to be attributed to structures of greater volume such as the rER, after quantitative analysis. However, since there is no method

available for determining which of the values are more accurate, it is unsure which of the results derived using either the 230 nm circle or the nil circle are underestimates or overestimates. The impression is that the large circle creates underestimates of diffuse structures and those comprising little relative area, while the use of the nil circle presents an overestimate for this type of organelle (i.e., membranes). On the other hand, the reverse seems to be true for numerous relatively small tubular or ovoid structures, such as the mitochondria. Since mitochondria are spread extensively throughout cells and are usually in close proximity to many other structures, grains shared over mitochondria and another organelle are more likely to be attributed to the mitochondria if a large circle is used, and less likely with a small or nil circle. Thus, it is expected that before analysis, the interpretation of the distribution pattern of silver grains would be quite different for two such different circle sizes.

However, after analysis by the method of Nadler, minor variations in data were not found to be significant by χ^2 for observed values where $p = 0.05$ (Table II-2). Since essentially the same results are achieved by direct scoring of the structure under the centre of the grain as the radioactive source, as those derived by analysis with a boundary circle equivalent to the resolution of ^{55}Fe (HR = 204 nm; Parry & Blackett, 1973), then perhaps a complex quantitative method may not be necessary.

The results derived by the method of Blackett & Parry, as used in the present study, support this rationale and emphasize the likelihood that direct scoring of grains, that is using a nil resolution boundary circle and thus eliminating further analysis, provides similar results to those found after analysis with a 230 nm circle. Using this method though, there

were significant differences found between the 100 nm circle when compared to both the 230 nm and 0 nm circles. The effect caused by shared grains is especially notable in the case of the mitochondria, where a small circle resulted in an appreciable underestimate, while the larger circle provided data that was greater than that found using the nil circle. Again though, regardless of insignificant variation, results derived with the 230 nm circle after analysis were not statistically different from those found with the 0 nm circle without further manipulation. This would indicate that the use of a boundary circle and subsequent analysis could be eliminated without adverse effects on quantitation.

Hypothetical Tissue Observations

The use of cell models and the assumption of a uniform distribution of radioactive label allowed comparisons of data to be made, for one, on the basis of the percentage variation of individual results from an *a priori* unit concentration. *A posteriori*, there were no significant differences between the average percent variations from unit concentration for both circle sizes (Table III-4), thus indicating that the margins of error encountered by using different circle sizes were not statistically different. In addition, if the average variation is used as an index of accuracy (Table III-3), the evidence shows that results found using the 230 nm circle deviate from unit concentration by a mean of about 50%, when all organelles are included. If small organelles (i.e., < 10% of total area) are omitted, then this falls to about 25 to 30%. If the average variation observed using the 230 nm circle differs by 25 to 30% from that derived using the nil circle, then the inverse statement should be true. That is, using a nil circle will result in an average variation of about 25 to 30% from data

derived using the 230 nm circle. In the case of heavily labelled structures, with many shared grains, or those making up a large proportion of the volume, the absolute difference may appear significant. For example, a value of 80% of the relative content attributed to an organelle by a nil circle seems remote from a value of 60% attributed to the same structure by a 230 nm circle after analysis (i.e., 25% variation). For smaller structures with less radioactivity, this difference will likely be negligible; for example, 8% of the label attributed by the nil circle compared to 6% for the 230 nm circle (i.e., still 25% variation). Thus a practical measure of accuracy is introduced to the concept of using a nil resolution boundary circle based on the determination of hypothetical grain distributions as observed in these hypothetical cell models.

Comparisons of individual composites, using different circle sizes, indicated that for the majority of cases the results did not differ significantly (Tables III-6 to III-10a). Where there were differences, these were shown to be attributable to relatively small structures (e.g., mitochondria, Golgi), especially where their distribution in the hypothetical tissue was diffuse (Table III-11).

When data for the composite tissue standards are analysed collectively as the average relative content of label in individual organelles from all composites (Table III-12), again, no significant differences are observed between results found using both circle sizes or the nil circle, except where cross-reference comparisons are made between the small circle of one method to the large circle of the other (Table III-12a). This evidence supports the notion that the size of the resolution boundary circle, within the limits of practical use, does not have as important an effect on the interpretation

of grain distributions as has been previously suspected. Moreover, the employment of a boundary circle may not be necessary at all, since results derived by direct scoring (nil circle) are essentially identical to those found after analysis with a 100 nm or 230 nm circle.

The Methods of Analysis

Experimental Tissue Observations

In regards to the study of thyroid tissue after administration of ^{55}Fe, the results derived by both methods of analysis were comparable (Table II-1), and found to be not significantly different when circle sizes involved were the same (Table II-2). When the small circle of one method is compared to the large circle with the other method, then there are differences due almost entirely to variations observed for relative content of label in the mitochondria. Except for this, most other values compare well between methods and give a statistically good fit with the χ^2 test for observed values (Freund, 1962).

The method of Blackett & Parry establishes the standard error of the mean (±S.E.M.) for these data and exhibits the probable range of these values (Fig. III-B). The χ^2 comparisons made during the course of these experiments did not take into account the standard errors calculated by the Blackett & Parry computer analysis. Rather, comparisons of the data were made using the mean amounts of relative content derived by this method with those derived by the method of Nadler. For example, in regards to the cross-reference comparisons of data from the small circle of one method to the large circle of the other, if the standard errors were included, then the apparent differences became diminished. The content derived for the rER by the Blackett & Parry method (65.8%) with the small circle is

dissimilar from that derived by the method of Nadler (45.4%) with the large circle. However, if the standard error of ± 18.6% is included for the Blackett & Parry data, the values fall close to within the same range, indicating that they would probably not be statistically different, especially if the S.E.M. was included for the method of Nadler as well.

In view of the fact that the 230 nm resolution boundary circle was closer to the size suggested by Parry & Blackett (1973) under similar conditions for ^{55}Fe, the observation that results derived by both methods especially for this circle size are virtually identical, would indicate that any differences seen in the final data have not been caused by the methods. The method of Nadler showed a tendency towards underestimating the amount of label in smaller organelles, while overestimating that in larger ones (see Table II-1), but these differences were not significant (Table II-2).

Therefore, by experimental investigation, under the specific conditions described, it has been demonstrated that the two methods of analysis are equivalent. Perhaps of greater interest is the interpretation that directly scoring the structure underlying the centre of the grain as the exclusive radioactive source resulted in essentially the same distributions of labelled content as those established after analysis by both quantitative techniques.

Hypothetical Tissue Observations

Both methods showed similarities when compared in relation to their percentage variation from an assumed unit concentration of radioactive label (Table III-3). The method of Nadler displayed little, if any, difference from that of Blackett & Parry, except when using the 100 nm circle, where the variations were slightly less than under other conditions. In essence, both methods were equally accurate (or inaccurate), and no significant

difference was observed between them by the χ^2 test for observed values ($p = 0.05$) as shown in Table III-4. The method of Nadler appeared to underestimate predicted contents about 75% of the time, while that of Blackett & Parry produced equal proportions of underestimates and overestimates (50% each; see Table III-5). The underestimation by the method of Nadler was usually caused by smaller structures being attributed with less label than might be expected. With the Blackett & Parry method, there is no indication as to whether predicted values will be over or under in their estimation of relative amounts of radioactivity. With this method, it may be assumed that there is an equal probability that the predicted value for relative content will be either greater or less than the actual value which should be attributed to a particular organelle.

In reference to comparisons of individual composites, about 30% of all the results derived by the 100 nm circle showed significant differences between the methods. For the 230 nm circle, about half of the data of all composites were significantly different (15 out of 33) as seen in Table III-9. In both situations, though, most of the differences were attributable to variations caused by the small size or extensive diffusivity of an organelle, especially for the mitochondria (Table III-9a) since these almost always involved a relatively small proportion of the labelling (< 10%) the importance of these differences may be negligible. In addition, rather than exposing differences in the techniques, these variations were interpreted to indicate that while the size of the resolution boundary circle reflects the probability of including radioactive structures within limits of the grain, it does not take into account the diffusivity of structures, which is dependent upon an entirely different distribution pattern. While both analytical methods do

include systematic investigation of the shape and distribution of organelles, these are based on the size of the boundary circle being used in the study. Altering the circle size will have an effect on the interpretation of the diffusivity of organelles, since ultrastructural distributions are independent of the resolution of the radioautographic technique.

Comparisons of average relative content from all organelles established by both methods (Table III-12) revealed no significant differences between them, except when the 100 nm circle used with the Blackett & Parry method was compared to the 230 nm circle used with the method of Nadler (Table III-12a). Again, it became apparent that the method of Nadler tended to predict underestimates for smaller structures, with the greatest difference seen in association with the mitochondria. However, the distributions were not significantly different (by χ^2) when analysed as a group.

The data derived by direct scoring was not significantly different from results established by either method (see Table III-12a). The impression is that both methods are equally valid, and equally accurate for the quantitative analysis of electron microscope radioautographs. In addition, the practice of direct quantitation without further manipulation appears to provide results which are not statistically different from those derived after application of extensive analytical procedures.

Applicability of the Methods

In light of the fact that under the specific conditions involved, the results derived using both methods were found to be equivalent, they can now be compared in relation to their applicability to an experimental investigation. If put into use, both methods require a resolution boundary circle of a size reflecting the probability with which a radioactive source

will be included in the circle, when superimposed over a grain. This probability may be derived on theoretical grounds or by experimental observation.

Both methods require the use of transparent templates for determining relative areas of organelles and distributions of relative content of radioactive label (Figs. II-1 to II-4). Both require statistical manipulations which can be carried out with the aid of an electronic computer. On this point, however, there is a difference in convenience between the techniques. The method of Nadler offers the option of rapid calculation by a computer program in Basic language, designed for the Hewlett-Packard desk-top calculator (Model 9810A) and as well, the applicable formulae for manual computation by numerical analysis, which can be accomplished with the assistance of a non-programmable hand-held calculator. The method of Blackett and Parry offers a computer program for pertinent calculations also, but computer time is presently available only through correspondence with the authors in Sutton, Surrey, England (Parry, 1976; Blackett & Parry, 1977).

Another difference is observed in the methodology of assessing relative areas of organelles. For the method of Nadler, these are established by the point-hit method (Chalkley, 1943), and comprise part of the input of the calculator program (See Fig. II-A, III-C). The method of Blackett & Parry includes relative areas as part of the output of its computer program after completing the analysis (Figs. II-B, III-D).

Since it has been shown that the quantitative analysis of E.M. radioautographs can be carried out by direct scoring, or with either the method of Blackett & Parry or Nadler, all producing similar results, it remains the option of individual investigators to choose which method best suits their own particular experimental conditions. The following guidelines

are suggested. If relative quantitation is not desired, or where a unique radioactive source is presumed and must be verified, then the line source method of Salpeter may satisfy necessary requirements. An example of this type of situation is where the label is suspected over the cell surface and no other organelle, as observed for initial binding sites of neurotransmitters such as acetylcholine, at the neuromuscular junction.

Where quantitation is preferred, several approaches may be taken. One may opt for direct scoring, or choose one of the methods of Blackett & Parry or Nadler. From the results provided by these experiments, it was noted that the number of shared grains observed prior to analysis made up 28% of the total for the 100 nm circle radius in the study of thyroid tissue after administration of ^{55}Fe. The 230 nm circle attributed 53% of the grains as being shared over more than one organelle (see Discussion Table 1). From the data for the hypothetical cell models, a mean of 18.9 ± 6.8% of the grains were considered shared, while for the 230 nm circle this proportion was 36.2 ± 13.0% (see Discussion Table 2).

It is thus suspected that where fewer shared grains are observed by visual survey (e.g., less than 30% for a 100 nm circle radius or 50% for a 230 nm circle), then the method of direct scoring should provide results which are not expected to be significantly different from those derived by either analysis. Under conditions where direct scoring is intended, it will be necessary to determine relative areas of organelles by additional stereologic survey (e.g., Chalkley, 1943; Loud, 1962; Weibel, 1969). Further mathematical calculations will naturally be required in order to derive relative content and concentration values.

Where the number of shared grains is greater than about 30% for a

circle of 100 nm radius, or about 50% for a radius of 230 nm, then either of the methods of Blackett & Parry or Nadler may be considered. Since the choice of the size of the resolution boundary circle is essentially arbitrary, then a range within the limits of about 100 nm to 230 nm radius should not have a significant effect on the results. It must be expected though, that the incidence of shared grains will be higher for a larger circle radius (230 nm) than for a smaller one (100 nm), as shown in Discussion Tables 1 and 2.

If in an electron microscope radioautograph the distance between the perimeters of possible labelled structures is greater than twice the radius of the chosen resolution boundary circle, then this would effectively omit the need for a circle at all, since grains over these structures should be expected to be exclusive to individual organelles. On the other hand, where almost all of the label is in small structures which are closer to each other than the distance allowed by the resolution of the radioautographic technique, no method can accurately discriminate between these possible sources. Therefore, any analytical procedures to interpret the radioautographic reaction must be expected to be, at best, only an estimate of the actual label in tissues.

DISCUSSION TABLE 1

Distribution of Exclusive and Shared Grains observed in Experimental Tissue of Rat Thyroid Follicles 4 Hours after Administration of ^{55}Fe in vivo (Tissue supplied by Dr. S. A. Cassol)

As determined by the procedures of the Method of Nadler prior to statistical analysis, with a 100 nm and 230 nm radius resolution boundary circle

		Circle Size	
Exclusive Grains		100 nm	230 nm
Nucleus	(N)	18	17
rER	(R)	77	54
Mitochondria	(M)	13	1
Golgi	(G)	4	1
Membranes	(Mb)	14	5
Colloid	(C)	17	15
Lysosomes	(L)	2	1
Apical Vesicles)	(Av)	11	7
Exclusive Total:		156	102
Shared Grains			
N + R		6	9
N + G		0	0
R + M		28	54
R + G		2	6
R + Mb		7	23
C + R		1	1
C + Av		3	3
R + L		8	10
C + Mb		7	10
Shared Total:		62	116
Exclusive + Shared Total:		218	218
Shared grains as a percentage of total:		28%	53%

DISCUSSION TABLE 2

Percentage of Shared Grains observed in Hypothetical Tissue Standard Composite Photographs

As determined by the procedures of the Method of Nadler prior to statistical analysis

Assessed with a 100 nm and 230 nm radius resolution boundary circle

Hypothetical Tissue Standard Composite No.	Percentage of Shared Grains Circle Size	
	100 nm	230 nm
1.	19	30
2.	18	34
3.	18	35
4.	22	43
5.	16	34
6.	17	41
7.	23	40
8.	14	35
9.	14	29
10.	22	38
11.	19	34
12.	18	35
13.	15	30
14.	21	37
15.	20	43
16.	16	39
17.	12	28
18.	10	20
19.	17	31
20.	14	29
21.	18	35
22.	19	43
23.	19	37
24.	23	42
25.	45	82
26.	24	47
27.	35	65
28.	8	16
29.	18	33
30.	22	43
31.	17	41
32.	12	34
33.	12	26
Average % for all composites ± S.D.	18.7 ± 6.8	36.2 ± 13.0

SUMMARY AND CONCLUSION

The technique of employing a radioactive line source specimen for interpretations of radioautographs was examined in order to study the effects of various physical parameters on the resolution of the radioautographic method. The implications of these effects on the size of the resolution boundary circle, which is necessary for certain quantitative analyses, was investigated in relation to the application of the two main quantitative techniques currently in use, that of Nadler and of Blackett & Parry. Both methods were used to determine grain distributions using a small, 100 nm radius resolution boundary circle and a large one of 230 nm radius. These results were compared to those derived with a 0 nm circle where there are no shared grains, thus eliminating the necessity for further statistical analysis. This was carried out by performing an experimental study of the distribution of radioactive ^{55}Fe, four hours after administration to rat thyroid follicles (provided by Dr. S. Cassol) and by an examination of various (33) hypothetical tissue standards or cell models, produced with the aid of biological specimens, photography and computer-assisted randomization.

Data were interpreted to indicate that both the methods studied were equally accurate in their estimation of predicted results. In addition, no significant difference was observed between circle sizes in the majority of the cases studied. There was also no significant difference found between both methods and results derived using the 0 nm circle. Since the proportion of shared grains in most cases was less than 50%, it is contended that under similar circumstances, the practice of direct scoring is expected to produce results that would not be statistically different from those produced

after extensive quantitative analysis.

In the future, as further technological advances are made regarding reduction in the size of the silver bromide crystal in the emulsion, the size of the developed silver grain in the radioautograph, and perhaps the eventual introduction of the ability to physically control the direction of the path of radioactive energy particles (as suggested by Caro, 1961), resolution of the radioautographic technique will still improve considerably. Nevertheless, for the present, the interpretation of silver grain distributions for the purpose of quantitation must still be made on an intellectual basis. It is anticipated that the evidence provided by these experiments will facilitate this task and aid in the determination of the location and amount of radio-labelled tracer molecules in experimental tissue sections prepared for quantitative electron microscope radioautography.

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