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MATHEMATICAL BACKGROUND TO STEREOLOGY AND MORPHOMETRY FOR DIAGNOSTIC PATHOLOGISTS

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Motto: To make a name for learning when other ways are barred, take something very easy and make it very hard.

ABSTRACT

A short introduction to the basic mathematical principles which govern stereology and morphometry is given. The selection of principles introduced was chosen to cover the needs of a pathologist active in diagnostic histopathology. For one to get an overall picture of the fields of morphometry and stereology also and acquaintance with statistics of sampling and potential computer applications is necessary.

INTRODUCTION

The mathematical backgound to stereology and morphometry has been studied by numerous authors (Weibel and Elias 1967, Underwood 1970, Weibel at al. 1972, Underwood et al. 1976, Chermant 1977, Weibel 1979, Williams 1980, Kalisnik 1981, although only a few recent ones have had diagnostic histopathology in mind (Collan and Romppanen 1982, Ahearne and Dunnill 1982, Baak and Oort 1983, Collan et al. 1983). At last morphometry is finding its way to where it should have been years ago - at the root of diagnostic decisions.

In this context one should realize that the elitist approach to stereology and morphometry, in which formulas appear more important than working applications, has hindered the wide acceptance of morphometric principles by diagnostic

pathologists, who have found such approach redundant, unpractical, unwise or even wrong. On the other hand mathematics cannot be avoided in this area even though the principles one should master are simpler than many of us have dared to think. In this paper we try to cover the aspects that we consider should be mastered by pathologists who would like to apply the principles of morphometry to their diagnostic work. These principles together with statistics and computer applications (Selkäinaho 1983, Oja and Collan 1983) underlie diagnostic morphometry, for example, in the diagnosis of metabolic bone diseases, borderline malignancy etc.

GEOMETRICAL PROBABILITY

In diagnostic histopathology it is extremely important to be sure that the lesion to be studied microscopically is present in the section given to the histopathologist. The histotechnician makes sure that the section includes the tissue. If the lesion in the sample is large the probability that it is present in the section is also large. If the lesion is small the probability that it is present in the section is small. This probability p is dependent on the thickness H of the lesion in the direction at right angles to the plane of cutting and the thickness S of the sample in the same direction (Fig. 1)

$$(1) \quad p = \frac{H}{S}$$

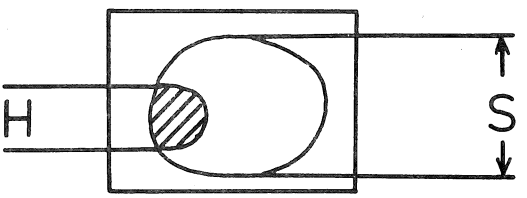

Fig.1

Exercise 1.

A pea has been embedded in a paraffin block. The thickness (s) of the block at right angles to the plane of cutting is 15 millimeters. The diameter (d) of the pea is 6 mm, which is also the thickness of the pea perpendicular to the plane of cutting. A random cut is made through the block. What is the probability (p) that the pea will be sectioned?

Solution:

$$p = \frac{H}{S} = \frac{d}{s}$$

$$p = \frac{6}{15} = 0.40$$

So the probability is 0.40, which means that in 40 sections out of one hundred the pea will be included in the section.

The above applies if the thickness of the section is not considered. If the thickness of the section t (which is transparent) is included in the calculations the probability is (Fig. 2)

(2) $$p = \frac{H + t}{S + t}$$

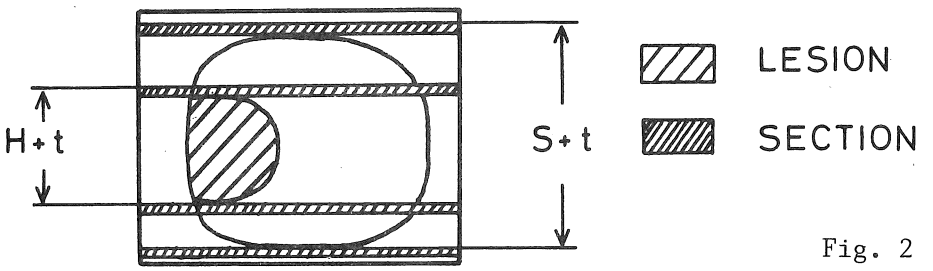

Fig. 2

Exercise 2.

A skin lesion, 2 mm in diameter, changed its colour from light brown to black in two weeks and started to bleed. The doctor suspected melanoma and excised the lesion. He made the excision with a 3 cm margin of normal tissue round the lesion. The sample was fixed in buffered formalin and sent to the pathology laboratory, where the fixed lesion was cut in 9 slices of equal thickness (Fig. 3) each of which was embedded in a separate paraffin block so that perpendicular cuts could be made. There was shrinkage of tissue during processing but as this was uniform throughout the sample it will not be considered here. We assume that the diameter of the lesion (H) perpendicular to the plane of cutting is 3 mm (a little larger than was macroscopically apparent). Sections 5 um thick are cut.

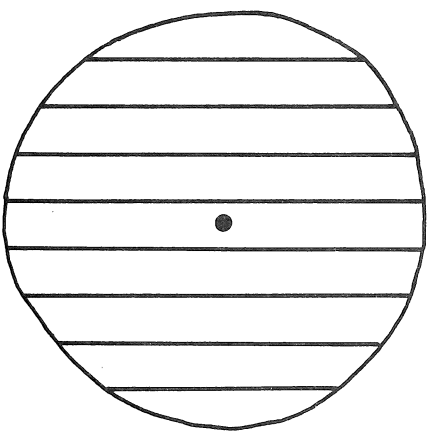

Fig. 3

A. In a block chosen at random what is the probability that the lesion is present in a random section?
B. In a block (f) which contains the whole lesion what is the probability that the lesion will be present in a random section?

Solution:

A. The diameter of the sample was 30 mm + 30 mm + 2 mm = 62 mm. So p can be calculated: (next page)

$$p = \frac{H + t}{S + t} = \frac{3\text{ mm} + 0.005\text{ mm}}{62\text{ mm} + 0.005\text{ mm}} = \frac{3.005}{62.005} = 0.048$$

So about 5 sections out of one hundred will contain the lesion or parts of it. Section thickness (0.005 mm) obviously does not markedly influence the result. So section thickness could be ignored in this example.
B. The thickness of each slice is 62/9 mm.

$$\text{So} \quad p = \frac{3 \times 9}{62} = \frac{27}{62} = 0.44$$

So about 44 sections out of one hundred will contain the lesion or parts of it. The thickness of sections is again ignored in this calculation.

The thickness of the sample or part of the sample in the direction perpendicular to the plane of cutting is called the caliper diameter and has the symbol H. If we have a sample which is not spherical and we change its orientation in the paraffin block the caliper diameter of the sample varies (Fig. 4).

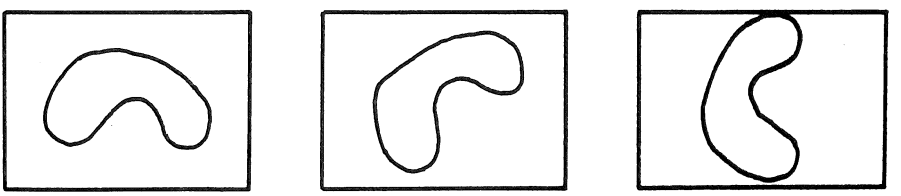

Fig. 4

In stereological investigations nuclei are often studied and when these are not spherical and their orientation varies, then the geometrical probability of finding an individual nucleus in the section also varies. If the nuclei are of constant size and shape and their orientation is random there is a mean caliper diameter (mean of the caliper diameters of all the nuclei in the sample) = H which can be used to estimate the average probability of finding a nucleus in the section.

Mean caliper diameters of standard geometrical bodies of the same size in a population with random orientation can be determined. In the case of spheres the mean caliper diameter is the same as the diameter of the spheres and some other values are as follows (from Underwood 1970):

- Cube 3a/2 a = edge of the cube
- Tetrahedron 0.9123a a = edge of the tetrahedron
- Octahedron 1.175a a = edge of the octahedron
- Pentagonal dodecahedron 2.57a a = edge of the dodechedron

- Cylinder 1/2 (r + h) h = height
  r = radius of the bottom
- Oblate spheroid (b/a = 1/2) 1.70 a
- Prolate spheroid (b/a = 1/2) 2.76 b
  a = longer axis of spheroid
  b = shorter axis of spheroid

(Oblate spheroid is formed when an ellipse rotates around its shorter axis, prolate spheroid when an ellipse rotates around its longer axis.)

Sometimes we have been surprised to find the lesion in one section but not in the other sections of the same sample. The probability p of finding the lesion in one section if it is present in another is dependent not only on the caliper diameter H, but also on the thickness of sections t_1, t_2, and the distance between the sections (s) (Fig. 5). In this situation we can apply the formula (Collan and Collan 1970)

$$p = \frac{H - s}{H + t_1 + t_2 + s}$$

Fig. 5 shows how this formula is derived. The distance over which the cell is found in any one of the sections and the distance over which it is found in both sections are determined. The ratio of these distances is the probability in question.

This formula has been applied in situations in which successive sections have been studied with different methods and the findings related to individual cells or cell groups, for example sections from the same block cut both for electron microscopy and light microscopy, or sections from the same block stained differently to show that certain proteins are or are not secreted by the same cells or same group of cells.

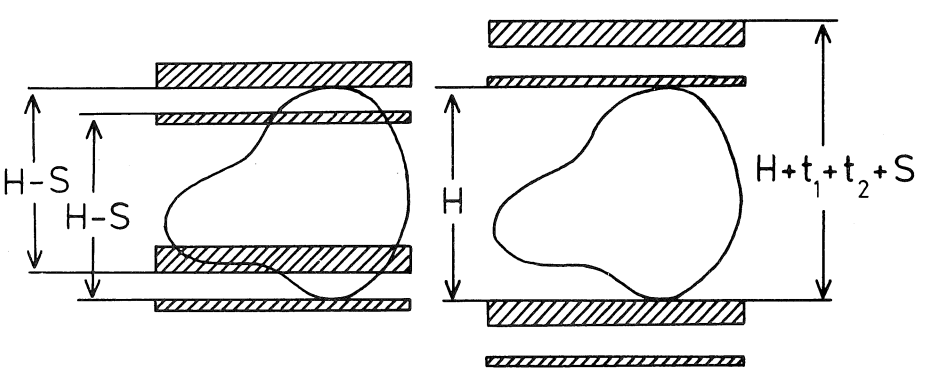

Fig. 5

Exercise 3.

Tritiated thymidine was injected into the peritoneal cavity of a mouse. After an hour the mouse was killed. Samples were taken from the gut mucosa and prepared for electron microscopy. One 1.0 μm thick section and 3 ultrathin sections (0.06 μm thick), were cut from the Epon-embedded tissue. The 1 μm thick section was covered with photographic emulsion which was exposed for 3 weeks and thereafter developed and fixed. The tissue was stained with methylene blue and the section and the autoradiograph above it inspected with a light microscope (Collan 1973). Many lymphoid cells in the lamina propria had grains above their nuclei which suggested active DNA synthesis. Sections through the centers of these cells suggested that the cells were about 8 μm and their nuclei about 5 μm in diameter. What is the probability that such a cell or its nucleus would be found in the third ultrathin section cut after the 1 μm thick section if the cell is found in the latter?

Solution: The relevant parameters are shown in Fig. 6.

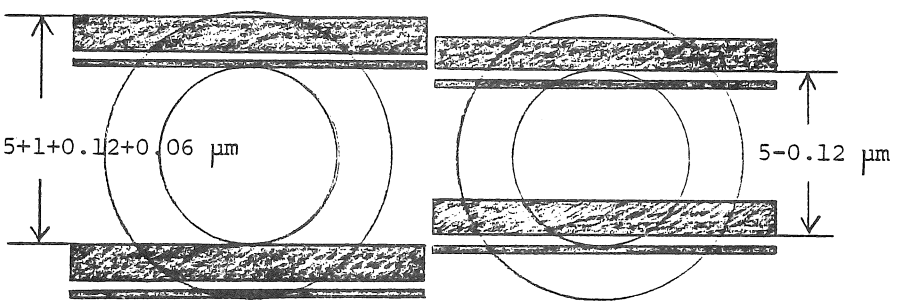

Fig. 6

The probability in case of the whole cell will be:

$$p = \frac{H - S}{H + t_1 + t_2 + S} = \frac{8 - 0.12}{8 + 1 + 0.06 + 0.12} = \frac{7.88}{9.18} = 0.86$$

So the cell will be present in the third ultrathin section, after it has been found in the light microscope section, 86 out of one hundred times.

In the case of the nucleus the corresponding probability is

$$p = \frac{5 - 0.12}{5 + 1 + 0.06 + 0.12} = \frac{4.88}{6.18} = 0.79$$

In some cases the thickness of the sections turns out to be very important in morphometric analysis. In such a situation one should be able to define thickness. This may be

complicated. It suffices to say here that microtome settings and scales are not always reliable, and interference colours of ultrathin sections give a rough estimate. Williams (1980) discusses instruments which can determine the thickness of sections reliably. In another paper Williams (1981) covers the available method for paraffin, semithin and ultrathin sections.

VOLUME FRACTION

Volume fraction V_V is the ratio of the volume of a particular tissue component (Vi) and the tissue volume which contains the tissue component (Vr). Here the concept tissue component includes numerous particles of identical character or a large continuous component possibly only partly included in the volume studied. To cover both of these alternatives we usually speak of tissue phase. So,

$$(4) \qquad V_V = \frac{Vi}{Vr}$$

DELESSE PRINCIPLE

Delesse, a French geologist of the 19th century, studied random sections of rock. He measured the area covered by a tissue phase on the section and related it to the area of the section. This ratio is the area fraction A_A of the tissue phase (Fig. 7). It can easily be shown that, if a very large number of sections are studied, the volume fraction and the corresponding area fraction are indentical. If fewer sections are studied the results are estimates of V_V. How good these estimates are is determined by statistical sampling theory, the principles of which can be applied in this context. This is usually the point at which the probabilistic and statistical nature of stereology emerges during a morphometric study.

So we can state that, for a very large number of random sections of tissue,

$$V_V = A_A$$

For small number of sections

$$(5) \qquad V_V \approx A_A \quad ,$$

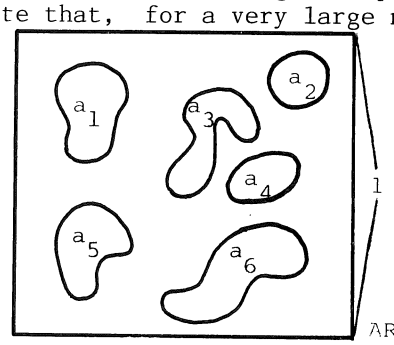

Fig. 7

and the section(s) can be considered a sample of the tissue in question.

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ESTIMATION OF AREA

Numerous methods can be used to estimate the area of tissue on the section. Such estimations are necessary for determining the area fraction A_A.

The microscopic image can be photographed, enlarged and printed on photographic paper, or the tissue phase of interest can be drawn on paper after the image has been projected on it. The areas can then be determined by planimetry with an instrument called planimeter or by cutting the tissue out and weighing it. Also the reference volume should be determined by planimetry or weighing. Magnetized digitizer tablets can also be used. Various kinds of grids can be used. For example one can superimpose a line grid randomly on the photograph or picture of the image and determine the lengths of the lines covering the tissue phase under study - let the summed length be Li. When the length of the lines of the grid on the section is Lr,

$$L_L = \frac{Li}{Lr}$$

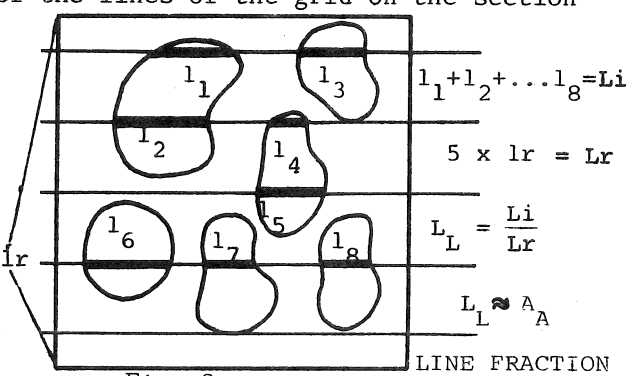

Fig. 8

It has been shown that in area estimation

(6) $A_A \approx L_L$

especially in cases in which the total length of lines covering the tissue phase is great.

Most often, however, point grids are used. The number of points falling on the tissue phase in question and the total number of points studied, Pr, give us the point fraction, P_P,

$$P_P = \frac{Pi}{Pr}$$

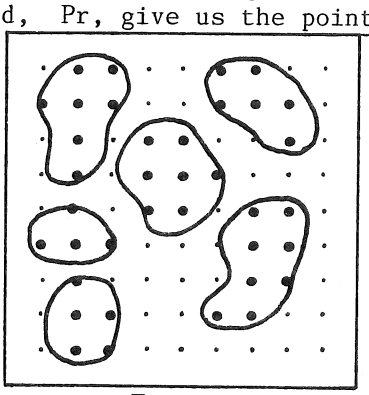

Fig. 9

It has been shown that

(7) $A_A \approx P_P$,

and this correspondence is true especially in situations in which the number of random sections is large and the number of points falling on the tissue phase under study is large. Again the probability that this estimate of Aa will be within a certain confidence interval of the true Aa can be estimated by applying the principles of sampling theory.

We may also point out that in computerized image analysis, in which each picture is digitized into numerical form, the estimation of Aa is done efficiently by a computer (Oja and Collan 1983).

Exercise 4.

A test grid was randomly laid on a microscopic image. The grid contained 600 points and covered an area of 122500 um^2. 183 points were on cell nuclei, the rest were on cytoplasm or interstitium. Give a good estimate for the volume fraction of the nuclei in the tissue studies.

Solution: $$V_V \approx P_P = \frac{183}{600} = 0.305$$

A good estimate for the volume fraction is 0.31.

LENGTH DENSITY

Sometimes it would be most interesting to get an idea about the total length of a meandering linear or tubular structure. For this it is necessary to know the number of intersections of the structure in the test plane, i.e. the number of sectioned profiles of the structure per square unit of section. For this situation we have the equation

(8) $$L_V \approx 2 \times P_A$$

in which L_V is the length of line per unit volume of tissue (length density) and P_A is the number of intersections per area of the section.

Exercise 5.

The length density of capillaries in a tissue was studied. Cross sections and oblique sections (2 x thickness < length < 10 x thickness) were counted as one intersection each. Tangential sections (length > 10 x thickness) were counted as two sections. The result was 1600 intersections per mm^2 of section. What was the length density of capillaries in the tissue?

Solution: We apply the above formula (see next page)

$$L_V \quad 2 \times \frac{1600 \text{ intersections}}{\text{mm}^2} = \frac{3200 \text{ intersections}}{\text{mm}^2} =$$

We replace intersections with mm/mm and get

$$= \frac{3200 \times \text{mm}}{\text{mm}^2} = \frac{3200 \text{ mm}}{\text{mm}^3}$$

which shows that the tissue is richly vascularized.

SURFACE DENSITY

Randomly oriented surfaces, when cut in any plane, produce what looks like lines in the cutting plane. It is surprising but true that with a line grid one is easily able to estimate the surface density S (amount of surface per volume of tissue) of the sample. We do this by counting the intersections of the test grid lines and the lines produced by the sectioned surfaces (Fig. 11). Again a simple formula is available:

(9) $S_V \approx 2 \times I_L$

in which I_L is the number of intersections with the surface profile of test line.

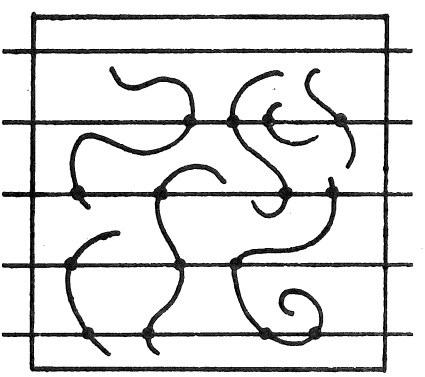

Fig. 11

Exercise 6.

Glomeruli were studied. On a photomicrograph of a glomerulus, magnified 100 times, a line grid was applied. Within the Bowman's space the test line length was 89 centimeters. The intersections of the test line and the line produced by the sectioned basement membrane (BM) were counted, the total number being 142. Give a good estimate of the surface density of the basement membrane within the Bowman's space.

Solution:

In this example we have added the magnifications to make the situation realistic. The idea in this approach is to transfer the data to the level of the sectioned glomerulus. Because the magnification was 1000, the test line length corresponds to 89 cm/1000 at the level of the glomerulus. This is 890 mm/1000 = 0.89 mm. Because there were 142 intersec-

tions, I_L was 142 intersections/0.89 mm. So we can go further:

$$S_V = 2 \times \frac{142}{0.89} \times \frac{\text{intersections}}{\text{mm}} = 319 \frac{\text{intersections}}{\text{mm}}$$

We replace intersections with mm^2/mm^2 and get

$$319 \frac{mm^2}{mm^3} = 319 \text{ mm}^2 \text{ BM/mm}^3 \text{ of Bowman's space}$$

The above formula for surface density (S_V) is based on the fact that the length of line produced by the sectioned surface in the section, is directly related to the surface density according to the formula

$$S_V \approx 4\, l_A / \pi$$

where l_A = is the length of the line per unit test area (Weibel 1976). This "areal length density" l_A, on the other hand, can be measured by superimposing a line grid on the photomicrograph according to the formula

$$l_A \approx \frac{I \times \pi}{2L}$$

in which L is the total length of the grid line and I is the number of times the grid lines cut the profile of the surface. When the above formulas are combined, we get

$$S_V \approx \frac{4}{\pi} \times \frac{I \times \pi}{2L} = \frac{2I}{L} = 2I_L$$

which is formula (9) above.

NUMERICAL DENSITY

The average number of sectioned profiles of a tissue component per area of section (N_A) is not enough for the calculation of numerical density (number of component bodies in volume, N_V). What is also needed is the mean caliper diameter ($\bar{H}$). In fact it is not difficult to show that

$$(10) \qquad N_V = \frac{N_A}{\bar{H}}$$

It would be easy to determine N_V if only $\bar{H}$ could be easily determined. But there is the problem. Determination of the mean caliper diameter of tissue bodies is problematic because assumptions on shape and size distribution are necessary. We return to this point below.

If section thickness is also considered in the calculation the above formula changes to

$$(11) \qquad N_V = \frac{N_A}{\bar{H} + t}$$

where t = thickness of the section

Exercise 7.

Herring eggs, of mean diameter 0.8 mm, were embedded in paraffin. In a 10 µm section there was a mean of 55 eggs per square centimeter. What is the number of herring eggs in a cubic centimeter (= 1 milliliter) of the paraffin block?

Solution:

$\frac{N_A}{\bar{H}}$ = 55 eggs/cm^2
$\bar{H}$ = 0.08 cm
t = 0.001 cm

$$N_V = \frac{55 \text{ eggs}}{\text{cm}^2 \ (0.08 \text{ cm} + 0.0001 \text{ cm})} = \frac{55 \times 1000 \text{ eggs}}{81 \text{ cm}^3} =$$

679 eggs/cm^3

If the thickness of the section is not considered the result is

$$N_V = \frac{55 \text{ eggs}}{\text{cm}^2 \times 0.08 \text{ cm}} = \frac{55 \times 10000}{80 \text{ cm}^3} = 688 \text{ eggs/cm}^3$$

The difference between the results is about 1% of the result. Also the ratio $t/\bar{H}$ is about 1%. In practice this difference is negligible. The thickness of the section can be ignored when the caliper diameter of the component studied is this much larger than the thickness of the sections.

Ebbeson and Tang (1967) recommended the use of two section thicknesses for this kind of analysis. Under the conditions of such an experiment shape and size assumptions are no longer necessary, nor do we need to know the caliper diameter. The only problem in practice is that it is difficult to cut sections of certain definite thickness and to determine the thickness of a section (Williams 1980, 1981). The formula presented by Ebbeson and Tang is: (next page)

$$(12) \qquad N_V = \frac{N_{A1} - N_{A2}}{t_1 - t_2}$$

where N_{A1} and N_{A2} are the mean numbers of profiles per area in the transparent sections and t_1 and t_2 are the respective thicknesses of the sections. The formula follows directly from the formula (11) above.

Exercise 8.

Two paraffin sections of the same homogeneous tissue were cut, one was 5 µm and the other 15 µm thick. Using light microscopy the number of nuclear profiles per square millimeter was calculated. In the 5 µm section the result was 1650 nuclei/mm^2 and in the 15 µm section 2500 nuclei/mm^2. What is the number of nuclei per cubic millimeter in the tissue?

Solution:

$$N_V = \frac{2500 \text{ nuclei/mm}^2 - 1650 \text{ nuclei/mm}^2}{15 \text{ µm} - 5 \text{ µm}} =$$

$$\frac{850 \text{ nuclei} \times 1000}{\text{mm}^2 \times \text{mm} \times 10} = 85000 \text{ nuclei/mm}^3.$$

Correction for "lost caps". If the objects to be studied in histological sections stain lightly they can be seen easily only if they occupy the whole thickness of the section. Usually these objects are spherical or spheroidal. If the cap of such an object is in the superficial layers of the section, it might be impossible to detect the object. If the thinnest caps are lost from detection estimates of N_V will be too low. Floderus (1944) worked out a correction formula for this situation:

$$(13) \qquad N_V = \frac{N_A}{H + t - 2h}$$

where h is the depth of the cap (perpendicular to the plane of the section) at the limit of detection.

Exercise 9.

Rytömaa (1960) estimated the numbers of eosinophils present in rat tissues. The study was based on counting the number of cells per area of section. He estimated that the mean eosinophil diameter was 10 µm, and that a cap with a depth less than 0.5 µm was not observed. The section thickness

was 4 μm. In 6-month-old rats the mean number of eosinophils in paraffin sections of the spleen was 2.16 per 0.1 square mm. What was the number of eosinophils per cubic millimeter?

Solution: Floderus' formula is applied

$$N_V = \frac{2.16}{0.1\ mm^2\ (10 + 4 - 2 \times 0.5)\ \mu m} = \frac{2.16 \times 1000}{0.1\ mm^2 \times 13\ mm} =$$

$$\frac{2.16 \times 100000}{13\ mm^3} = 1662\ \text{eosinophils/mm}^3$$

(When the total number of certain cells in an organ or in the whole body is estimated, also the shrinkage, which is caused by tissue processing, is taken into consideration. Rytömaa did this in his study but we do not consider this here.)

If we are ready to make assumptions on the shape and size of tissue phase particles we call on several formulas. The first formula is that of Weibel and Gomez (1962). It considers constant shape particles and is shown below:

$$(14) \qquad N_V = \frac{K}{\beta} \times \sqrt{\frac{\bar{N}_A^3}{\bar{P}_P}}$$

in which K is a size distribution coefficient, β is a shape coefficient, $\bar{N}_A$ is the mean number of profiles per section area, and $\bar{P}_P$ is the mean point fraction of the sectioned profiles. At this level the reader is reminded that the point fraction is a good estimate of the volume fraction (for further details see Weibel and Gomez 1962, Underwood 1970, or Weibel 1979). This formula is not always simple to apply but as it has been applied in numerous contexts we give here an example of how the coefficients can be determined.

Exercise 10.

Romppanen (1981) studied the germinal centers of the chicken spleen. He wanted to determine the number of the germinal centers in whole spleens. To do this he first determined the number of germinal centers per volume of spleen tissue and applied the formula of Weibel and Gomez (above) for that purpose. He had to estimate the coefficients β and K. To estimate the shape coefficient Romppanen assumed that the germinal centers were prolate ellipsoids (see page 4) with a constant axial ratio - i.e. that the germinal centers were ellipsoids of the same shape. He estimated the mean axial ratio by measuring the mean axial ratio of germinal center profiles in sections. The mean axial ratio measured from

sections is not the same as the mean axial ratio in 3 dimensions, but was used as an estimate of it. He found the mean axial ratio to be 0.87 (SD 0.01). Weibel and Gomez (1962, see also Weibel 1979) have devised graphs with which one now can estimate β . In this case with the relevant graph β = 1.4. To estimate the size distribution coefficient we need to have an estimate of the mean size and its standard deviation. The factor K is the square root of the cubed ratio of the third moment to the first moment of the distribution:

$$(15) \qquad K = \sqrt{\left(\frac{D_3^3}{D_1}\right)}$$

Where D = third moment of the size distribution and D= first moment of the size distribution. What are these "moments" then? Moments are calculated statistics that characterize the population collected for a statistical study. Moments are of the form

$$(16) \qquad \frac{\sum_i f_i x_i}{n}, \quad \frac{\sum_i f_i x_i^2}{n}, \quad \frac{\sum_i f_i x_i^3}{n} \ \ldots \ \ldots \frac{\sum_i f_i x_i^r}{n}$$

Where f_i is the number of measurements x having the value x_i ; and n is the total number of measurements. $(\sum_i f_i x_i)/n$ is the first moment of the data, $(\sum_i f_i x_i^2)/n$ is the second, $(\sum_i f_i x_i^3)/n$ is the third etc. The measurements x can be chosen in different ways. They can be the absolute value, i.e. the value of the observations, or difference of any kind - usually the difference between the mean and the observed value. If x is defined in the latter way, then the second moment gives the variance of the data.

In our example we need the moments with zero as the reference - i.e. the moments are calculated with x being the absolute values of the data.

But before being able to calculate K, Romppanen had to get an estimate of the size distribution. To make this estimate he had to assume that the germinal centers were spherical. Earlier he assumed (in calculating β) that they were ellipsoids, but because easy solutions to size distribution problems of ellipsoids from two-dimensional sections are not available, the sphere is a convenient approximation. This was a good choice because the germinal centers were in fact roughly spherical. He measured the diameters for a number of germinal center profiles (arithmetic mean of the longest semiaxis and the longest semiaxis perpendicular to the longest semiaxis), arranged their size distribution within 10 size classes and then transformed the data to 3-dimensional size distribution using Wicksell's method (1925). Thereafter K could be read from a graph published by Weibel and Gomez (1962).

Weibel (1979) has pointed out that in making estimations on biological variables K can often be disregarded. If we deal with objects of about the same size this is true. If we have a normal distribution of size parameters with a standard deviation of 25 % of the mean, K = 1.07. In many biological situations K will be between 1.02 - 1.1, and often arbitrary constants could be used. If the size distribution is wider or if the experimental situation has changed the size distribution, the determination of K by experimental means (as suggested above) may turn out to be necessary.

Also the method of de Hoff (1964) is based on shape assumptions and deals with constant shape particles:

$$(17)\quad N_V = \frac{N_A^2}{2I_L} \times \frac{\gamma_2}{\gamma_1^2} \quad \text{or}$$

$$(18)\quad N_V = N_A \times \frac{2I_L}{P_P} \times \frac{\gamma_1\ \gamma_2}{\gamma_3}$$

where I_L is the mean number of intersections of test lines and profile boundary per the length of test lines on the image, and γ_1 , γ_2 , γ_3 are shape coefficients which are dependent on the shape of the particles studied. For further details see de Hoff (1964) or Weibel (1979). Also Aherne (1967) and Loud (1968) have given methods which can be applied for determining numerical density.

PARTICLE SIZE DISTRIBUTION

If spheres of varying sizes are sectioned, it is not easy from the section to get an idea about their size distribution in three dimensions. One can start by drawing a size distribution of sectioned spheres in the section, and by estimating the mean diameter of the sectioned spheres by Fullman's (1953) formula. In this formula N is the total number of circular profiles measured, and d_1 .....d_N are the diameters of the profiles. The mean diameter D of the spheres is

$$(19)\quad D = \frac{\pi}{2} \times \frac{N}{\frac{1}{d_1} + \frac{1}{d_2} + \frac{1}{d_3} + \ldots \frac{1}{d_N}}$$

If one needs more detailed information, other approaches are necessary. In the following we are only treating spheres and refer to the graphical method by Elias and Hennig (1967) to show the basic principles. This method is easy to understand, can be easily computerized and has given good results in practice (Baak et al. 1977).

The method starts by defining the size distribution of the circular sections of spheres in the tissue sections. After measurements we get a histogram of a certain number of size classes. With the help of the histogram we can draw a smoothed out size distribution curve. We assume that we get the following curve (Fig. 12):

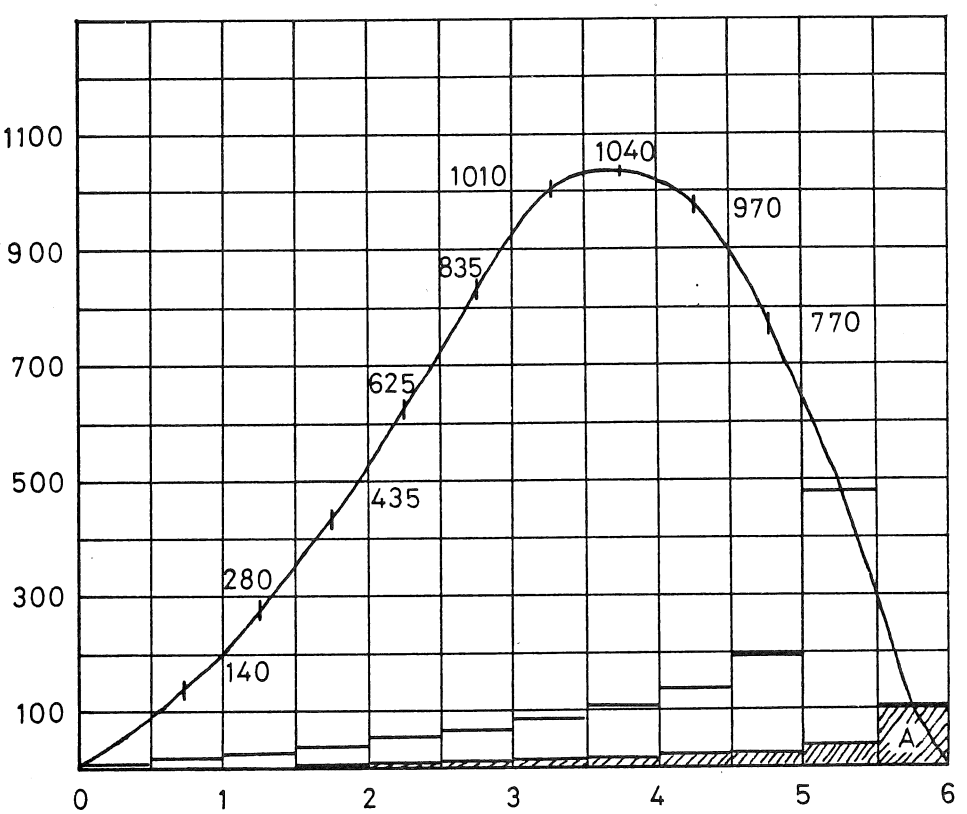

Fig. 12. The smooth curve presents the size distribution of circles on a plane which cuts across a population of spheres. The circles cut from the largest size group of spheres are shaded. A presents the largest circles of that group. Horizontal lines show the contribution of the spheres from the second largest size group (5.01 - 5.50 units) above the shaded histogram. The figures refer to the number of circles at the points marked by vertical dashes on the curve. The figures are the sums of contributions from the various size groups of spheres. These figures are also shown by column totals in Table 2.

Now we decide how many size classes are convenient in the 3-dimensional reconstruction. Let us take 12 size classes of 0.5 units. We also assume that in the size group 5.51 - 6.00 units we deal only with the spheres with the real diameter of 6 units. This means that all circular section profiles in the same size range are sections of spheres with the diameter of 6 units. Because we know the number of the largest sections now we can easily estimate how many sections of smaller diameter groups are also cut across 6 unit diameter spheres in the sample. Because the spheres have been cut randomly, the laws of geometrical probability define the numbers of cuts of each size class. The largest number of sections will be near 6 units across, thereafter the number of sections decreases as the size decreases. The number of sections in each size class can be graphically estimated as shown in Fig. 13 and the numbers entered under the curve into the column of each size group. This has been done in Fig. 12 for the largest two size groups of spheres.

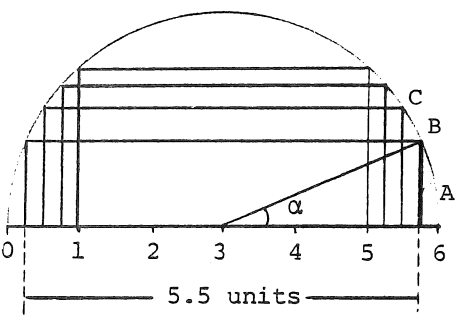

Fig. 13

If graphical reconstruction turns out to be laborious we can also apply trigonometry. In Fig.13 A corresponds to a distance of 3 units times the sine of angle α . Because we know cos α (lower limit of the size class divided by 2 and 3 = 5.51/(2 x 3)), we can determine sin α easily, and the fraction of profiles of each size generated through cutting 6 unit spheres can thereafter be calculated. For the case in Fig. 13 we can first calculate the ratio of 5.51/6 = 0.917 (which is cos α) and use a calculator to find the corresponding angle, i.e. we determine arc cos α . The sine of this angle is the fraction of the radius (3 units) corresponding to the height A, also easily determined with a calculator or trigonometric tables. The other fractions (B,C.. in Fig. 13) can be determined in a similar fashion by first determining the sine values of the corresponding angles and subtracting from these the sines of the former size classes. The data can then be listed as in Table 1, which shows the fractions of sections in each profile diameter size class after identical spheres have been cut a random. This table helps us further as will be shown in the example below. A corresponding table can be generated for any sizes of spheres by applying the following computer programme. The programme is written in BASIC for a Sinclair ZX-81 computer.

Program for ZX-81 "Slicing spheres"

```
10 PRINT "GIVE THE RADIUS OF THE SPHERES"
```

```
 20 INPUT A
 30 LET E = 0.5
 40 LET B = (A - E)/A
 50 LET C = ACS B
 60 LET D = SIN C
 70 PRINT D
 80 LET SUM = D
 90 LET E = E + 0.5
100 LET B = (A - E)/A
110 LET C = ACS B
120 LET D = SIN C
130 IF (D - SUM)<=0 THEN STOP
140 PRINT D - SUM
150 GOTO 80
```

In the program, E = size of class interval, B = cos a, and ACS B = arc cos a.

The next group of sections (5.01 - 5.50 units) is assumed to consist of sections across spheres of the largest diameter (6 units) and of spheres across the next smaller size class of spheres. We assume that in the next size class the spheres are all of 5.50 units in diameter. We now see that the rest of the circular sections in the size group of 5.01-5.50 units in Fig. 12 consists only of cuts of spheres of the diameter 5.50 units. Again when we know the number of largest cuts, we

TABLE 1

The fractions of sections (circles, 2-dimensional) in 12 diameter size classes after spheres (3-dimensional) with diameters of 0.5-6.0 units were cut at random. Thickness of sections is not considered.

Diameter of spheres (3-D)	Fractions of sections in 12 diameter groups (2-D)											
	0.01 - 0.50	0.51 - 1.00	1.01 - 1.50	1.51 - 2.00	2.01 - 2.50	2.51 - 3.00	3.01 - 3.50	3.51 - 4.00	4.01 - 4.50	4.51 - 5.00	5.01 - 5.50	5.51 - 6.00
6.0	0.003	0.011	0.018	0.025	0.034	0.043	0.054	0.067	0.084	0.108	0.153	0.400
5.5	0.004	0.013	0.021	0.031	0.041	0.053	0.067	0.085	0.111	0.158	0.417	
5.0	0.005	0.015	0.026	0.037	0.050	0.066	0.086	0.114	0.164	0.436		
4.5	0.006	0.019	0.032	0.047	0.064	0.086	0.117	0.170	0.458			
4.0	0.008	0.024	0.041	0.061	0.085	0.119	0.177	0.484				
3.5	0.010	0.031	0.055	0.083	0.121	0.185	0.515					
3.0	0.014	0.043	0.077	0.012	0.193	0.553						
2.5	0.020	0.063	0.117	0.200	0.600							
2.0	0.032	0.102	0.205	0.661								
1.5	0.570	0.197	0.745									
1.0	0.134	0.866										
0.5	1.000											

can calculate or graphically determine the number of smaller cuts in the same fashion as in Fig. 13. Also the numbers of these smaller sections have been entered in Fig. 12. By continuing in this fashion we get an estimate for the three dimensional size distribution of spheres just by adding up the sections cut through each size of spheres.

Exercise 11.

In the above example, enter the number of sections of other size classes of spheres, or calculate their numbers, and finally determine the size distribution of the spherical bodies in three dimensions; and the mean and the standard deviation of that distribution.

Solution:

Enter the size classes as long as there is space under the curve. After more space is needed you have entered a size class which does not exist. Stop before that point. Now add up the sections cut of each size class. The graphical solution needs quite a long time and a lot of patience.

By applying the data in Table 1 one can also solve this problem but it is certainly better to draw at least some figures in order to understand what is going on. In Table 2 the numbers of sections within each size group (2-dimensional) are underlined. Under them (marked with an asterisk) are the numbers of sections in each size group after the sections generated by cuts through larger size spheres have been subtracted. The latter figures give the number of sections through the center of the size class of spheres in question. These figures have also been entered in the table columns (also marked with asterisks). As Fig. 12 and Table 2 show, there are 100 sections (2-D) in the size class 5.51-6.00. These are necessarily sections across spheres of 6 units in diameter. From Table 1 we can pick up the fractions of sections in other size groups by dividing 100 by 0.400 and multiplying it by the fraction of the 2-D-size group. For example, the number of sections in the 2-D-size group of 3.51-4.00 will be $0.067 \times 100/0.400 = 16.75 \approx 17$.

After the numbers of sections in each 2-D size class have been entered for 6.0 unit spheres, the 5.5 unit spheres are considered. Their number in size class 5.01-5.50 is 480-38 = 442, as shown in Table 2. The corresponding numbers in other size classes are entered thereafter; again using the data of Table 1 in the calculations. Because biological objects usually have a size limit below which there are no objects, figures are entered until the number of sections in the column exceeds the number of sections in the size group. The last sphere size

TABLE 2

List of numbers of sections in various size groups from spheres of various sizes. The list corresponds to the presented example and the numbers of sections (2-D) are those shown in the distribution of Fig. 8. The negative number in the column head of the size group 0.51-1.00 shows that spheres smaller than 1.5 units in diameter cannot be present.

	Diameter groups of sections (2-D), number of sections in each diameter group (underlined) and the number of sections in diameter groups after the number of sections from the larger diameter spheres has been excluded (asterisk).												
	0.01 - 0.50	0.51 - 1.00	1.01 - 1.50	1.51 - 2.00	2.01 - 2.50	2.51 - 3.00	3.01 - 3.50	3.51 - 4.00	4.01 - 4.50	4.51 - 5.00	5.01 - 5.50	5.51 - 6.00	
Diameter of spheres (3-D)	45 / -7	140 / -18	280 / 7*	435 / 41*	625 / 124*	835 / 268*	1010 / 451*	1040 / 554*	970 / 614*	770 / 576*	480 / 442*	100 / 100*	Totals
	Number of section profiles in each diameter class												
6.0	1	3	5	6	9	11	14	17	21	27	38	100*	252
5.5	4	14	22	33	43	56	71	90	118	167	442*		1060
5.0	7	20	34	49	66	87	114	151	217	576*			1321
4.5	8	25	43	63	86	115	157	228	614*				1339
4.0	9	27	47	70	97	136	203	554*					1143
3.5	9	27	48	73	106	162	451*						876
3.0	7	21	37	59	94	268*							486
2.5	4	13	24	41	124*								206
2.0	2	6	13	41*									62
1.5	1	2	7*										10
1.0													
0.5													
Totals	52	158	280	435	625	835	1010	1040	970	770	480	100	6755

whose figure has been entered is 1.5 units. But note that numbers of smaller sections reach higher values than could be expected - such error is very typical of this kind of analysis and is due to the shape of the distribution of 2-D section sizes. In this example the differences are not big in relation to the number of sections studied and this is why we can be satisfied with the size distribution shown in the right hand column of Table 2. From this data the mean and the standard deviation can be calculated.

The mean ($\bar{x}$) is:

$\bar{x}$ = (6 x 252) + (5.5 x 1060) + (5.0 x 1321) + (4.5 x 1339) + (4.0 x 1143) + (3.5 x 876) + (3.0 x 486) + (2.5 x 206 + (2.0 x 62) + (1.5 x 10) divided by 6755.

This gives $\bar{x}$ = 4.40 units.

The standard deviation is

$$SD = \sqrt{\frac{\sum N_i (x_i - \bar{x})^2}{\sum N_i}},$$

where N_i is the number of spheres in size class i and N_i is the total number of spheres sectioned. x_i is sphere size in size class i and $\bar{x}$ is the mean size of sectioned spheres. In other words (and figures) SD =

(6-4.4) 252 + (5.5-4.4) 1060 + (5.0-4.4) 1321 + ... + (1.5-4.4) 10, divided by 6755 and a square root taken of the result.

This gives 0.90 units.

There are also other methods of determining the size distributions of spheres. Several of these have been handled in detail in the books of Weibel (1979), Underwood (1980), Williams (1980) and Aherne & Dunnill (1982). The size distribution problem has been examined by Wicksell (1925, 1926), Schwarz (1934), Lenz (1954, 1956), Saltykov (1958), DeHoff (1962, 1965), Bach (1963, 1967) and Giger and Riedwyl (1970).

HOLMES EFFECT

This effect may affect the estimation of many stereological parameters. The Holmes effect is the change in the appearance of the image that is produced by finite section thickness as opposed to a "perfect" cut (section with no thickness). If we define the Holmes effect like this the effect will affect the results of N_V, A_A, V_V, and S_V and almost any other morphometric estimation.

Section thickness affects the results if sections are transparent and if there is enough contrast for a detail completely embedded inside the sample to show through the section. If the sections are transparent and the contrast is perfect this effect will play a part when the objects studied are not very large with respect to the thickness of the section. In V_V measurements the error caused by the Holmes

effect can be estimated with special formulas (Holmes 1921, Hennig 1967). The error exceeds 12 % of the result when the diameter of the object is less than 15 times the section thickness. If the diameter is less than 8 times the section thickness, then the error will exceed 20 %. Because perfect contrast is seldom attained one should be careful in applying these principles. Under less favorable conditions other sources of error may be important (see correction for "lost caps" on p. 7).

OTHER METHODS

Numerous other methods have been put forward in articles published in the field of stereology and morphometry and all of them cannot be covered in a single presentation. We therefore refer the reader to the more voluminous presentations of mathematical stereology already cited in the introduction. From the diagnostic point of view at least, parameters as "the nearest neighbour distance" and "connectivity" (Takahashi 1982) have potentially interesting applications.

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