PRACTICAL STEREOLOGICAL METHODS FOR MORPHOMETRIC CYTOLOGY

EWALD R. WEIBEL, GONZAGUE S. KISTLER, and WALTER F. SCHERLE

From the Department of Anatomy, University of Zürich, Zürich, Switzerland

ABSTRACT

Stereological principles provide efficient and reliable tools for the determination of quantitative parameters of tissue structure on sections. Some principles which allow the estimation of volumetric ratios, surface areas, surface-to-volume ratios, thicknesses of tissue or cell sheets, and the number of structures are reviewed and presented in general form; means for their practical application in electron microscopy are outlined. The systematic and statistical errors involved in such measurements are discussed.

INTRODUCTION

In a previous communication (49), we presented methods by which tissues can be examined quantitatively at the electron microscopic level of resolution. Further development of this work has yielded a set of methods which are widely applicable in the field of cytology and can efficiently produce a vast amount of reliable information. It is the purpose of this study to present these methods in practical form without entering into the theoretical considerations on which they are based; the latter have been treated extensively by various investigators to whom reference will be made in appropriate places.

Biological morphologists still shy away from quantitation of their results for the primary reason that direct measurement of structures on sections is very cumbersome and yields doubtful results. On the other hand, geologists and metallurgists, who are faced with problems similar to ours, have developed and are using methods of quantitation (12, 19, 8, 11, 42, 28) which, in recent years, have been applied to biomorphological problems with some applications touching on cytology (15, 21, 32, 44–49, 34, 40). With the development of reliable quantitative techniques in physiology and biochemistry, a correlation of structure and function of cells and tissues will necessitate the production of quantitative morphological data. The methods reviewed and presented here should assist this endeavor.

GENERAL CONSIDERATIONS

The internal structure of an organ can be investigated only after destruction of the tissues. The most commonly used method of *orderly destruction* in cytology is that of sectioning of fixed and embedded tissues. By sectioning, the integrity of the tissue is preserved in two of the three dimensions of space while the third dimension is sacrificed for the benefit of resolution. If the section thickness is very thin with respect to the size of structures investigated it can be disregarded and we can consider the section—which, in reality, is a slice of tissue—as a two-dimensional image of the internal tissue structure. Three features of such infinitely thin sections must now be noted (47):

1. Every structure of the tissue is usually cut at random by a nonoriented section.

2. An n-dimensional structure of the tissue is represented on the section by an $(n-1)$-dimensional image: bodies are seen as areas, surfaces as lines, and lines as points.

3. The quantitative occurrence of images on sections is determined by the quantitative presence of structures in the tissue sectioned. As an example, we mention the Delesse principle (12) which states that the planimetric fraction of a section occupied by sections of a given component corresponds to the fraction of the tissue volume occupied by this component. As we shall see, there also exist relations between the numer of structures in the unit volume and the number of transections of these structures found on the unit area of a random section (48). Such statements are valid in the statistical sense; i.e. the laws of error theory apply.

It is particularly this third feature of infinitely thin sections which enables us to use sections for quantitative morphological work (44). A large fraction of possible propositions in cytology can be so defined as to be conveniently solved by measuring volume, surface, and numerical densities in the unit volume. Surface-to-volume ratios are also a convenient measure which can provide a large amount of information.

In many instances, though, the finite thickness of the section may not be disregarded since it would introduce a systematic error. This will be dealt with below.

STATEMENT OF STEREOLOGICAL PRINCIPLES

A number of useful stereological principles and their application formulae will be stated here without proof or derivation; for all details, reference will be made to the original papers. All these principles have one common feature: They make use of random tissue sections of negligible thickness, on which a test system is placed at random. This test system is made up of "probes" in the form of points, lines, or planar areas, depending on the information sought.

These probes can either coincide with some particular features of the structure met on the section, or not coincide with them. The investigator has merely to count the positive events and can then derive from this finding the required information by appropriate application of the formulae.

A standard notation (42) will be used; the symbols are defined in Table I.

a. Volumetric Analysis (Fig. 1)

In 1846 the geologist Delesse (12) developed the fundamental relation of stereology, now generally named the Delesse principle, which states that the volume fraction V_{Vi} of a component i in the tissue can be estimated by measuring the area fraction A_{Ai} of a random section occupied by transections of i (Fig. 1 *a*):

$$V_{Vi} = A_{Ai} \quad (1)$$

In practical application, A_{Ai} had to be deter

TABLE I

Standard Notation for Stereology

(adapted from Underwood (42))

General Features

1. Symbols: Capitals refer to collective treatment of structures, lower case to individual structures; e.g. v = volume of individual, V = total volume of group of structures.

2. Subscripts: Capitals identify reference or test system, lower case identify the object.

Individual Dimensions

v_i	Volume of structure i.
s_i	surface area of structure i.
a_i	transection area of structure i on section.

Collective Dimensions of Group of Structures

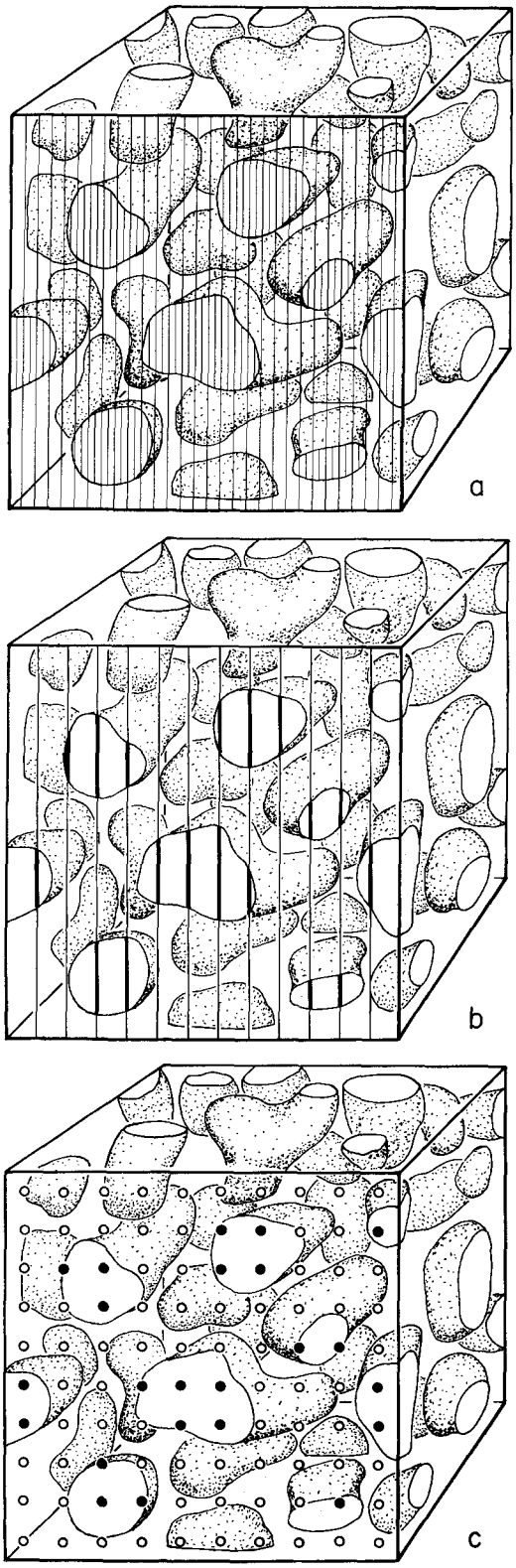
V_i	Collective volume of structures i.
S_i	Collective surface area of structures i.
A_i	Collective area of transections of i.
N_i	Number of structures i.
L_i	Collective length of intercepts of testline in i.
P_i	Collective number of test points lying in i.

Relative Dimensions

V_{Vi}	Volume fraction occupied by i (volumetric density).
S_{Vi}	Collective surface area per volume (surface density).
N_{Vi}	Number of i per volume (numerical density).
A_{Ai}	Area fraction of section occupied by i.
N_{Ai}	Number of transections of i per section area.
L_{Li}	Fraction of test line intercepting i.
N_{Li}	Number of intersections with i per test line length.
P_{Pi}	Fraction of test points enclosed in i.

Test Systems

V_T	Test volume.
A_T	Test area on section.
L_T	Length of test line.
P_T	Number of test points.



mined by cumbersome planimetry. Rosiwal (37) found, in 1898, that A_{Ai} could be estimated by lineal integration (Fig. 1 *b*), determining the lineal fraction L_{Li} of test lines passing through transections of i. This procedure is still in use (38, 34, 44). In final reduction of this principle, Glagoleff (19) found, in 1933, that planimetry could be done by superimposing a regular point lattice on the section and counting the points which lie on transections of the structures (Fig. 1 *c*). Chalkley (6) and Attardi (1) proposed similar procedures for application in histology. The fraction P_{Pi} of points lying on transections of i would thus be an estimate of V_{Vi}:

$$V_{Vi} = P_{Pi} \tag{2}$$

This procedure is the most efficient to use, particularly in electron microscopy (44, 49), since the necessary measurement consists merely of counting. Rather complicated analyses can thus be easily carried out (30, 46). Hennig (25) has also shown that point-counting volumetry is to be preferred to lineal integration, for statistical reasons. In the following, we will deal exclusively with point-counting volumetry.

As a practical example, we may wish to determine the relative volumes of nuclei (V_{Vn}), mitochondria (V_{Vm}), lysosomes (V_{Vl}), and cytoplasm (V_{Vc}) in particular cell types. A regular point lattice is placed at random on random electron micrographs of random sections. The information sought is obtained by differential counting of the points P_i lying on n, m, l, and c, whereby points lying on other structures (interstitium, etc.) are pooled in a compartment x. We obtain the total number of test points as

$$P_T = P_n + P_m + P_l + P_c + P_x = \sum P_i \tag{3}$$

and the volume fractions of the components as

$$V_{Vn} = \frac{P_n}{P_T};$$

$$V_{Vm} = \frac{P_m}{P_T} \text{ etc.}$$

FIGURE 1 Volumetric analysis of tissue composition on sections: (*a*) by planimetry; (*b*) by lineal integration; (*c*) by differential point counting.

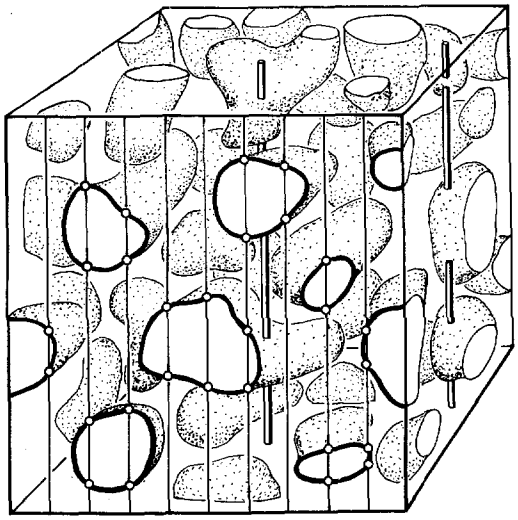


FIGURE 2 Determination of surface density from number of surface intersections with linear probes, which can be conveniently determined on sections.

generally by:

$$V_{Vi} = \frac{P_i}{P_T} = P_{Pi} \tag{4}$$

The absolute volume V_i of a given component in an organ, say of mitochondria in the liver, can be computed from V_{Vi} if the total volume of the organ V is known:

$$V_i = V_{Vi} \cdot V \tag{5}$$

b. *Measurement of Surface Areas (Fig. 2).*

Consider structures with a well defined surface embedded in the tissue. In the unit volume, these structures will occupy a certain relative volume or volume density V_{Vi}, as discussed above. Their total surface, S_i, included in the unit test volume, V_T, can be defined as their *surface density* S_{Vi}.

$$S_{Vi} = \frac{S_i}{V_T} \tag{6}$$

The surface of these structures will appear on sections as contour of the transections. The length of these contours on the unit test area of section will be proportional to the surface density, S_{Vi}. If a test line of fixed length, L_T, is randomly placed in the tissue it will pierce through the surface of these structures, whereby the number of intersections, N_i, between test line and surface will be proportional to S_{Vi} and to L_T. Tomkeieff (41) and Hennig (22) have independently derived the relation:

$$S_{Vi} = \frac{2 \cdot N_i}{L_T} = 2 \cdot N_{Li} \tag{7}$$

If the "surface" is considered to be a "sheet with two surfaces" the coefficient 2 has to be replaced by 4 (22).

Since the surface density, S_{Vi}, is represented on sections by a proportional "contour density," the test line can be placed at random on random sections of the tissue and N_{Li} can be obtained by simple counts of intersections between probe and surface contours.

As practical examples, the surface area of cells or of external and internal membranes of mitochondria can be determined by this method.

The total surface area of these structures in a given or known volume, for example in the organ volume, is obtained from

$$S_{iTOT} = S_{Vi} \cdot V \tag{8}$$

c. *Surface-to-Volume Ratios*

The surface-to-volume ratio of structures proves to be a very useful measure. On the one hand, it is related to geometric features of the structures, and, on the other, it can often serve as a direct measure of some physiological properties of these structures since it gives the size of their contact area with the surroundings per mass of structure.

Chalkley and Cornfield (7, 10) have derived a method for direct evaluation of the surface-to-volume ratio of structures: it is essentially a straightforward combination of point-counting volumetry with surface estimation by line intersection. The test system consists of short lines of equal length, Z, which again are randomly placed on random sections (Fig. 3). The two end-points of these test lines are used as markers for volumetry: end-points lying on sections of structures are counted as "hits" and recorded as P_i. Simultaneously, that is, without displacement of the test system, the number of intersections, N_{Zi}, of test lines and surface contour of the structures is recorded. The surface-to-volume ratio, s_i/v_i, of individual structures, i, follows from:

$$\frac{s_i}{v_i} = \frac{4 \cdot N_i}{Z \cdot P_i} \tag{9}$$

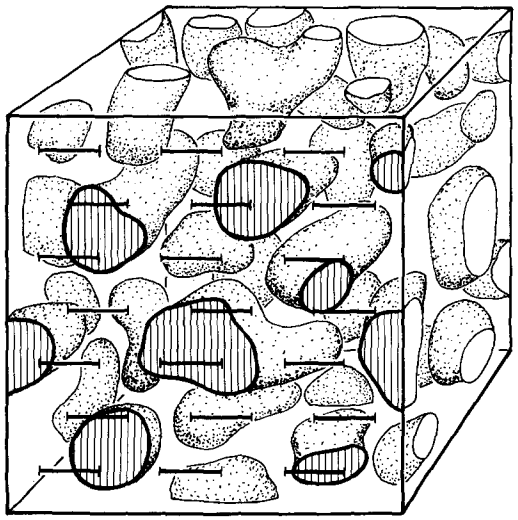


FIGURE 3 Estimation of surface-to-volume ratio with short linear probes.

This relation can be directly derived from equations 4 and 7 if we take $L_T = \frac{P_T}{2} \cdot Z$ as the total length of the test line, a condition which is fulfilled since there are two end-points to each short test line in the system of Fig. 3.

d. *Measurement of Average Thicknesses*

In a previous communication (49), we presented a method for estimating the arithmetic mean thickness $\bar{\tau}$ of a tissue sheet or a cell layer by modifying the above principle for surface-to-volume ratios. $\bar{\tau}$ is here defined as the average tissue volume per half the surface area of the sheet. Again, placing a test system of short lines of length Z on random sections of the sheet, i, we record, as above, the number of end-points, P_i, lying on sheet tissue and the number of surface intersections, N_i, and obtain

$$\bar{\tau} = \frac{Z.P_i}{2.N_i} \tag{10}$$

For considerations on transport across tissue layers, the harmonic mean thickness τ_h is often a more appropriate measure of the effective average thickness (44, 49). It is defined as the average of the reciprocals of all local thicknesses. Randomly placing long lines on sections of the sheet, we can record the intercept lengths l, that is, the length of test line lying between the two surface intersections, and form the harmonic mean intercept length, l_h, by averaging the reciprocal values of l. From this we obtain

$$\tau_h = \frac{2}{3} l_h \tag{11}$$

e. *Estimation of the Number of Particulate Structures*

The number N_{Vi} of particulate structures i contained in the unit volume of tissue can again be estimated from sections (Fig. 4). We found that the number of transections of i counted on the unit area of random section, N_{Ai}, is proportional to N_{Vi} (48, 44). However, this relationship is not straightforward. It depends on a variety of factors which have to be determined by other means. Most importantly, it depends on a knowledge of the shape of the structures; there are various means of determining this from the shape of section images (14, 16). In addition, we need to know something about the size distribution of structures (11, 31, 4).

If the structures studied are all of the same shape and roughly of the same size, two basic relations between N_{Vi} and N_{Ai} are available. De Hoff and Rhines (11) have shown that

$$N_{Ai} = N_{Vi} \cdot \bar{D}_i \tag{12}$$

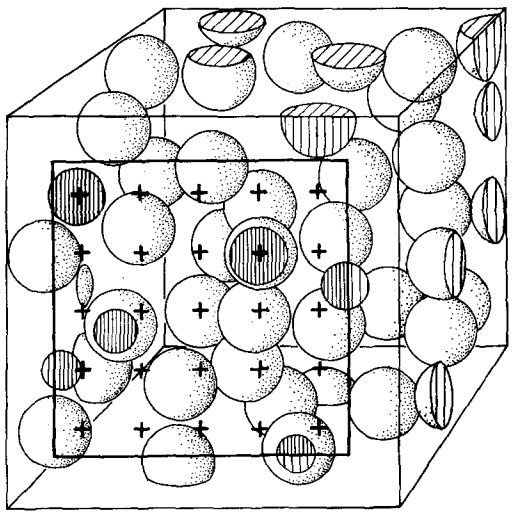


FIGURE 4 Estimation of number of (spherical) structures from number of transections and volumetric density.

where D_i is the average "tangent diameter" of the structures. In the case of spherical structures, $\bar{D}$ is simply their average diameter. For ellipsoidal structures, $\bar{D}$ can be determined graphically by the method of Bach (4).

While a knowledge of shape and size of the structure is necessary for application of this method, another principle which was derived by Weibel and Gomez (48) requires only a rough knowledge of shape. This is introduced into the relation between N_A and N_V in form of a dimensionless coefficient β. Values of β for typical bodies have been presented in graphic form (48). In addition, we need to know the volumetric fraction V_{Vi} occupied by the bodies; this can be obtained by simple point-counting analysis. The following formula applies:

$$N_{Vi} = \frac{1}{\beta_i} \cdot \frac{{N_{Ai}}^{3/2}}{{V_{Vi}}^{1/2}} \tag{13}$$

N_{Ai} and V_{Vi} can be determined simultaneously if a test system is chosen in which a lattice of points is enclosed in a square area (Fig. 4). We count the number of transections, N_{Ai}, lying within the square (counting those transections which are cut by the left and upper margin and rejecting those cut by the right and lower margin), and determine the point fraction, P_{Pi}, lying on transections according to equation 4. In equation 13, we then can substitute P_{Pi} for V_{Vi} and obtain the number of structures in the unit volume if we can make some assumptions with respect to their shape (β_i).

This latter principle can also be applied if the size of the structures varies within certain limits. A size distribution coefficient K must then be introduced (31, 45):

$$N_{Vi} = \frac{1}{\beta_i} \cdot \frac{{N_{Ai}}^{3/2}}{{V_{Vi}}^{1/2}} K \tag{14}$$

K is the ratio of third to first moment of the distribution of diameters (31). It is thus always larger than 1. For a normal distribution with a standard deviation of $\pm 25\%$ of the mean, $K = 1.07$.

Well defined biological structures often show a rather narrow size distribution. For renal glomeruli it was found, for example, that $K = 1.014$ (31, 45). This coefficient can thus be disregarded for many practical purposes or replaced by an arbitrary coefficient of the order of 1.02 to 1.1, particularly when comparisons between control and experimental materials are sought. Caution is indicated, though, when an experiment may have induced a change in the size distribution; this can be tested by determining the variance of the size distribution of the sections in both control and experimental materials (3).

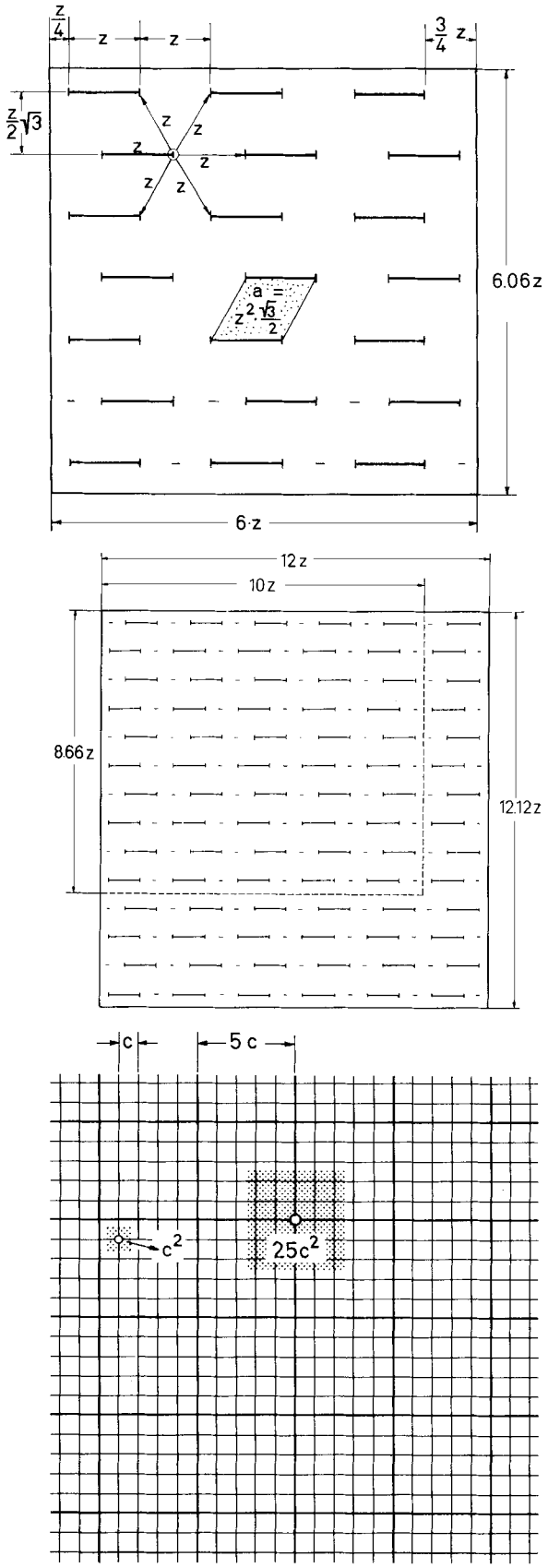
A MULTIPURPOSE TEST SYSTEM

The presentation of the above principles has shown that morphometric work requires the following "probes": a *test area* for counting structures, a lattice of *test points* for volumetric work, and *test lines* for surface estimation. A number of test systems have been proposed for various tasks (6, 7, 1, 20, 25, 44, 45, 49), and it is essentially a matter of personal preference which one is selected for a particular problem. If volumetric analysis of tissues alone is wanted at the light microscope level, the Zeiss Integrating Eyepiece I may be used (23). Similarly, for surface area measurement in the light microscope the Zeiss Integrating Eyepiece II is suitable (22). For counting transections any eyepiece containing a reticle with a square grid can be used.

In our own work, a multipurpose test system, which combines area, points, and lines (Fig. 5), was found to be particularly useful since it enabled us to obtain a large variety of information with one and the same test system. It was developed from a system presented previously for work done directly on the screen of the electron microscope (49). We are using three variants which differ only in the number of test lines and test points, but not in the general properties.

Fig. 5 *A* shows the general arrangement of the test system. A square area contains 21 lines of constant length, Z, which are arranged on 7 equidistant and parallel rows, whereby the distance between the end-points of the lines is Z in every direction. These $P_T = 42$ end-points thus form an equilateral triangular lattice; they can be used as test points for point-counting volumetry. With this arrangement each test point has an absolute area value $a = \frac{\sqrt{3}}{2} \cdot Z^2$; it is thus possible to perform planimetry with this test system as well (1, 27), by ascribing to each point lying within the planar area to be measured this area value a.

The $\frac{1}{2} P_T = 21$ test lines of length Z serve as probes for surface estimations according to equation 7. By recording N_L as the number of inter-



sections of these test lines with surface contours *per test field*, the total length of the test line is

$$L_T = \tfrac{1}{2} P_T \cdot Z$$

With this test-system it is also possible to determine surface-to-volume ratios according to equation 9, and average thicknesses of tissue layers according to equation 10.

The test-system is enclosed in a square frame whose sides measure 6 Z; it encloses an area which corresponds to nearly $P_T = 42$ area values of the test points. If one side of the frame measures 6.06 Z (Fig. 5 *A*), this relationship is exact. This frame defines the test area, A_T, for counting the number N_A of particle sections per test area used for determination of the number of structures in the unit volume N_V according to equations 12, 13, or 14. It is thus possible to obtain with this test system all measurements described above, except for the harmonic mean thickness τ_h. The test system illustrated in Fig. 5 *A* is particularly useful for light microscope work. It is commerically available incorporated into a measuring eyepiece,[1] whereby it can be directly superimposed with histological sections. For electron microscope work, it is desirable to have a denser arrangement of test points and lines. Fig. 5 *B* illustrates two variants of this test system which have the same properties, but differ inasmuch as there are 4 times more points and lines ($P_T = 168$) in the field; the sides of the enclosing square measure 12 Z. The dotted line encloses a test system with $P_T = 100$ which is convenient by giving directly per cent values for volumetry. The enclosing test area is a rectangle with the horizontal side measuring 10 Z and the vertical side

$$5 \cdot \sqrt{3} \cdot Z = 8.7 \cdot Z$$

[1] Manufactured by Wild Heerbrugg Instruments Inc., CH-9435, Heerbrugg, Switzerland. (No. 5678).

FIGURE 5 Stereological test systems. *A*, Multipurpose test system with 21 lines of length z arranged in equilateral triangular network of lattice period z. (compare text). *B*, Extended multipurpose test screens with 168 or 100 test points. *C*, Square lattice with double-lattice period of c and 5 c for volumetric analysis involving scarce component.

ALTERNATE TEST SYSTEMS

If volumetry alone is required it may be desirable to use a simpler test system. Haug (20) and others have used simple square grids, where the cross-points of lines serve as test points. Hennig (23, 25) has proposed hexagonal point-nets and has devised the Integrating Eyepiece I of Zeiss for their application.

In studying the volumetric density of scarce components in the cytoplasm, it may be advantageous to use a double-lattice test system as shown in Fig. 5 *c*. It consists of a square network with a lattice constant c, whereby every fifth line is heavier than the intermediate lines. The cross-points of all lines have an area value c^2; i.e., planimetrically each point represents one little square of c^2. The heavy lines alone enclose squares of area $(5\ c)^2$; the area value of the cross-points of the heavy lines thus equals $25\ c^2$. The relative volume, V_S, of the scarce component s in the volume of the cytoplasm, V_C, can be obtained by counting the number P_s of cross-points falling onto s and the number P_c' of *heavy* cross-points falling onto cytoplasm, through the formula

$$\frac{V_s}{V_c} = \frac{P_s}{25 . P_c'} \tag{16}$$

With this test system, it is possible to greatly reduce the counting labor without appreciably reducing the quality of the result. On the contrary, the error probability is reduced since we can choose a point density with which at least one test point will fall onto every section of s. Modifications of this principle can easily be found to suit every problem. A particular example is given below.

PRACTICAL APPLICATION OF THESE PRINCIPLES

Light Microscopy

It has already been pointed out that various test systems can be optically superimposed on tissue sections in any ordinary microscope. Methods for random sampling of tissue sections and of microscope fields have been presented previously (44). In recent work we have made extensive use of very thin sections of plastic-embedded material (30, 46) viewed with a phase contrast microscope.

Electron Microscopy

In previous work (49), we used a test system engraved into the fluorescent screen of our electron microscope and thus directly obtained the necessary measurements. We have now found it advantageous to record the electron micrographs on 35-mm film and to evaluate contact prints of these negatives in a table projector. This procedure is more efficient, reduces the necessary microscope time to a minimum, and ensures better resolution. The sequence of steps is described below.

A. SAMPLING: The application of the aforementioned stereological principles depends on sound random sampling of the material. The tissue is cut into the customary dices which are fixed and processed in bulk. A random sample of, e.g., 10 dices is drawn (44); these are consecutively numbered and embedded in individual capsules without orientation, and the sections are mounted on copper grids fitted with a carbon-enforced Formvar film.

The sections are randomly subsampled in the electronmicroscope. Fig. 6 shows a low power electron micrograph of a 150-mesh copper grid carrying a section of rat lung. Random subsamples of such sections can be obtained by recording electron micrographs in one specific corner of each mesh. In the case of Fig. 6, the circles mark the position of the viewing screen; randomness is achieved by letting the edge of the screen just touch two edges of the copper bars. Depending on the number of electron micrographs required, one can either start with an arbitrary field and record all consecutive frames until the sample size desired is reached; or, the fields to be recorded can be located by means of random numbers as proposed previously (44). The first procedure is less cumbersome, however, and is adequate for most applications. The fields selected by one of these methods are recorded on 35-mm film. At the end of each recording period, a test specimen (carbon grating) is micrographed for calibration purposes. The developed film strip is contact-printed onto positive film in a long light-box.

The square frame inside the circles marks the field finally recorded on the film and represents the sample studied. It should be noticed that the amount of lung tissue contained in each sample field varies considerably. If information on the composition of the entire organ—including air, tissue, and blood—is sought, all the samples ob-

tained have to be subjected to subsequent analysis, regardless of their content.

B. PROJECTOR UNIT: For evaluation of the positive film strips, we are using a small table projector which was constructed in our workshop (Figs. 7 and 8). A Leitz Pradix 35-mm projector (*P*, Fig. 8) fitted with a 50-mm objective and a 35-mm film stage (*F*) projects the picture via a mirror (*M*) onto a screen (*S*). The mirror is mounted with a friction hinge; millimeter paper is projected from film stage onto screen and the mirror tilt adjusted until the picture shows no distortion.

The screen consists of a semitransparent acetate paper with the multipurpose test system (Fig. 5 *b*) drawn in india ink; it is inserted between two glass plates. It is thus easy to exchange the test systems according to the requirements of the work. It is also possible to use suitable microfilm-reading devices which are commercially available.

C. EXAMPLES OF MORPHOMETRIC ANALYSIS: For each particular problem studied, it will

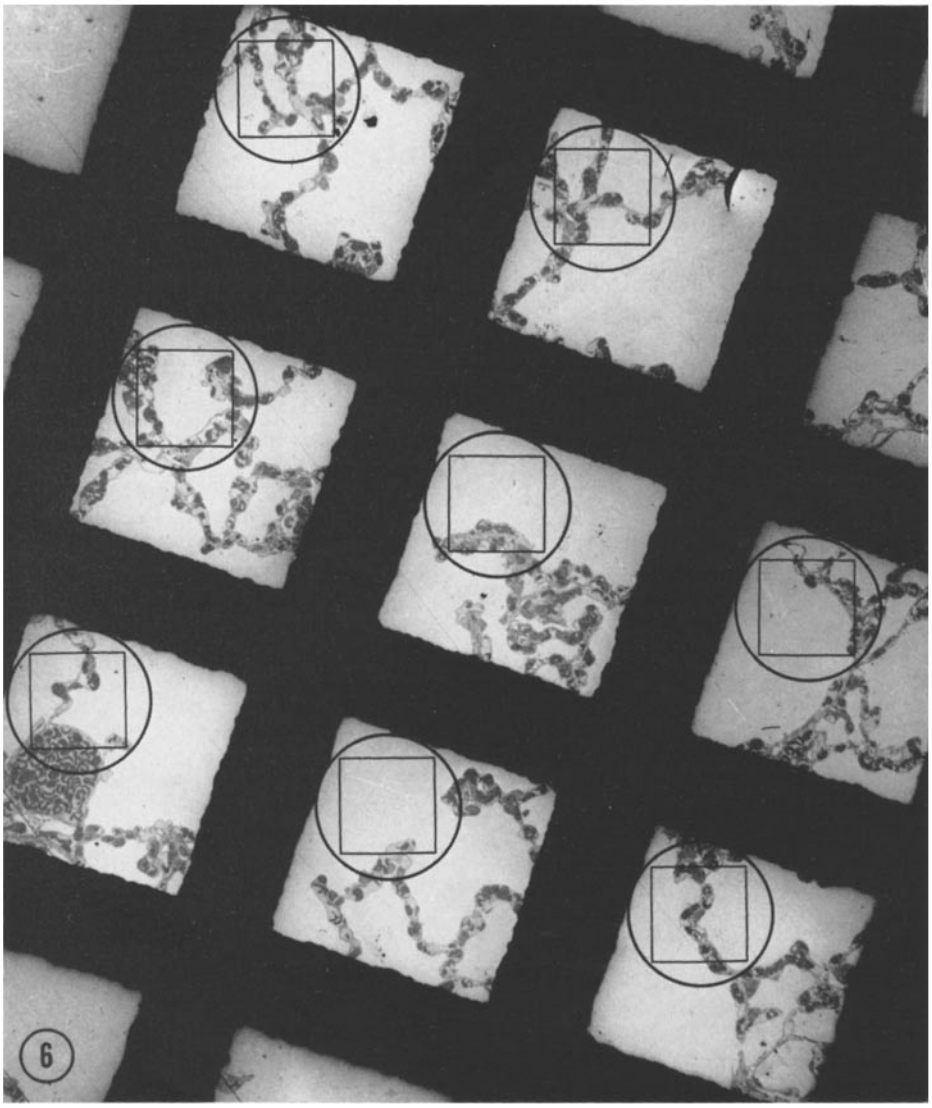


FIGURE 6 Electron micrograph of coppergrid carrying rat lung section. Positioning of screen (circles) in corners of grid for random sampling of fields. × 250.

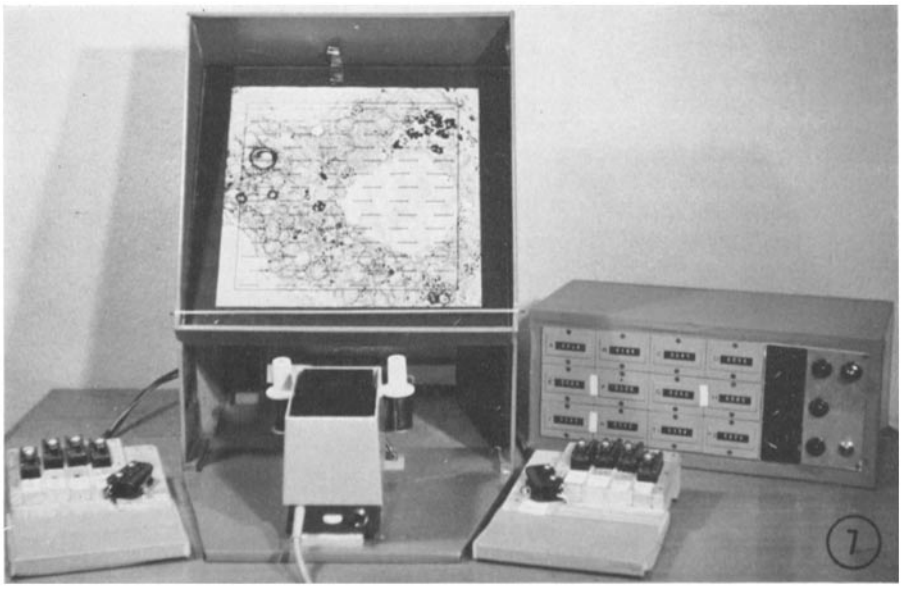


FIGURE 7 Assembly of projector and counter units with keyboards for stereological analysis of electron micrographs.

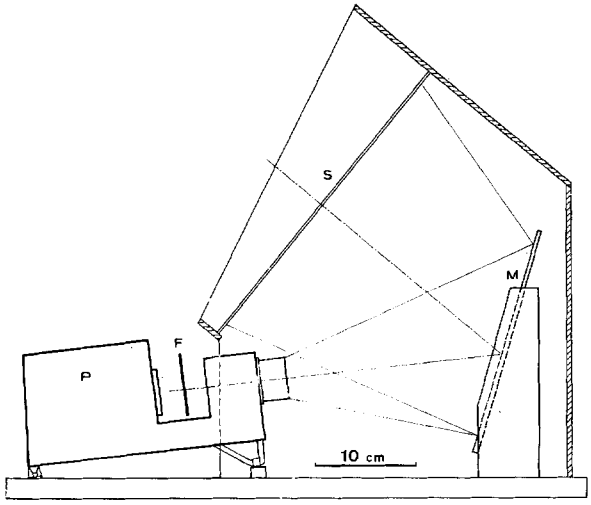


FIGURE 8 Diagram of table projector unit, with projector (P), film stage (F), tilting mirror (M), and screen with exchangeable test system (S).

be necessary to find the most suitable test system and to precisely define the parameters sought. As mere illustrations, two examples from our own work will be given.

In a series of studies on the gas exchange apparatus of the lung (30, 46), we sought quantiative information on the total surface areas between air and tissue (S_a) and between tissue and blood in capillaries (S_c); in addition, the capillary blood volume (V_c), the air volume (V_a), the tissue volume (V_t), and the average thickness of the air-blood barrier ($\bar{\tau}$) had to be determined.

The necessary basic data were obtained in a single operation by projecting low power electron micrographs, as obtained by random sampling of sections according to Fig. 6, onto the multipurpose test system of Fig. 5 B. In Fig. 9, a typical field is superimposed with the test system. Starting from the left-hand upper corner, the location of each end-point of the test-lines on air space (P_a), tissue

(P_t), and blood (P_c) was tallied on a differential counter. Subsequently, the intersections of the test-lines with the alveolar surface (N_{za}) and with the capillary surface (N_{zc}) were tallied. Typical points are labeled on Fig. 9. It can easily be seen that, of the 168 test-points, 20 lie on tissue and 20 on blood; furthermore, $N_{za} = 26$ and $N_{zc} = 18$ in this sample. These data are tabulated and are used for calculation of the volumes and surface areas by means of appropriate formulas given above.

It is, of course, also possible to determine the volume proportions of epithelium, endothelium, and interstitium in the tissue space by tallying the point-hits on these components separately. The frame enclosing the test system can be used to count the number of erythrocyte transections in capillaries or of nuclear profiles per sample area.

In another study (18), we estimated the volume fraction of cytoplasm occupied by a new and specific organelle (50) of endothelial cells in different vessels of the rat. Systematic samples of endothelial cells were recorded on film at 6800 primary magnification. Projection onto a screen fitted with the double-lattice test system of Fig. 5 *C* yielded a final magnification of 61,200 diameters. Fig. 10 shows a fraction of the screen with a portion of endothelial cytoplasm containing several transections of the organelles. The cross-points of the heavy lines lying on cytoplasm were counted

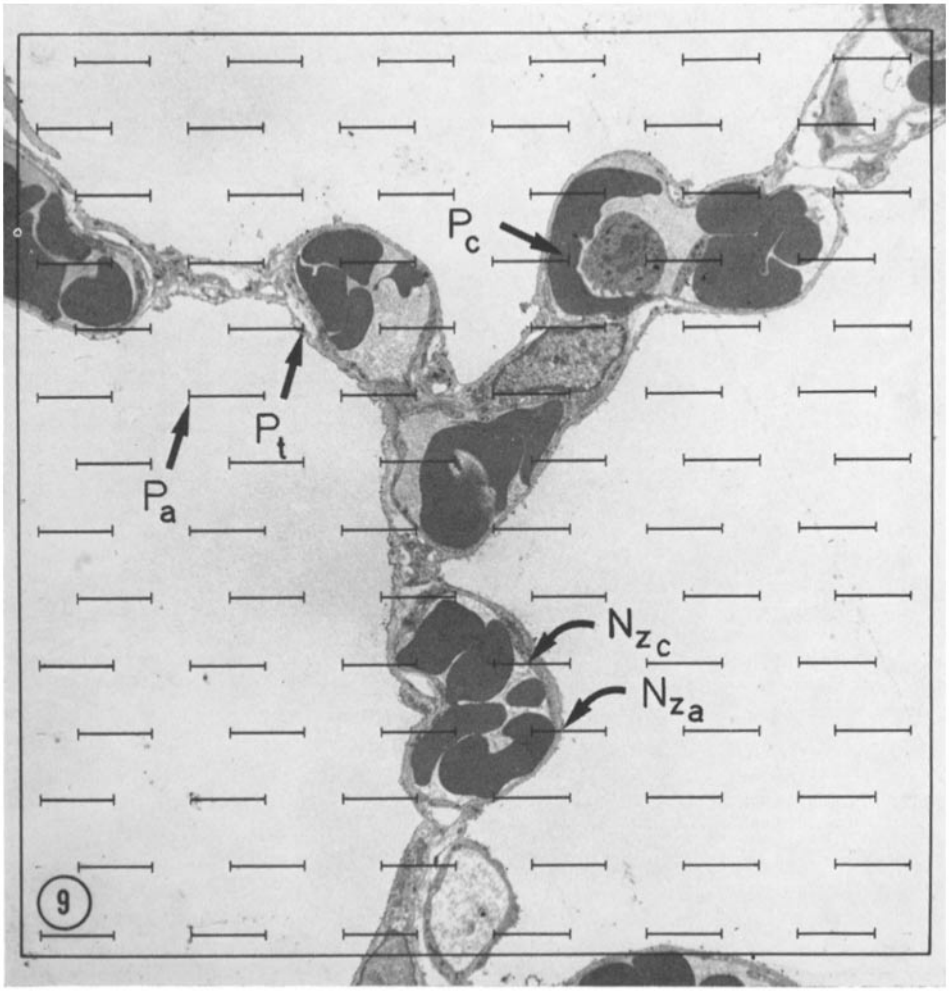


FIGURE 9. Sample field of rat lung section superimposed with multipurpose test-system. Compare text. × 2500.

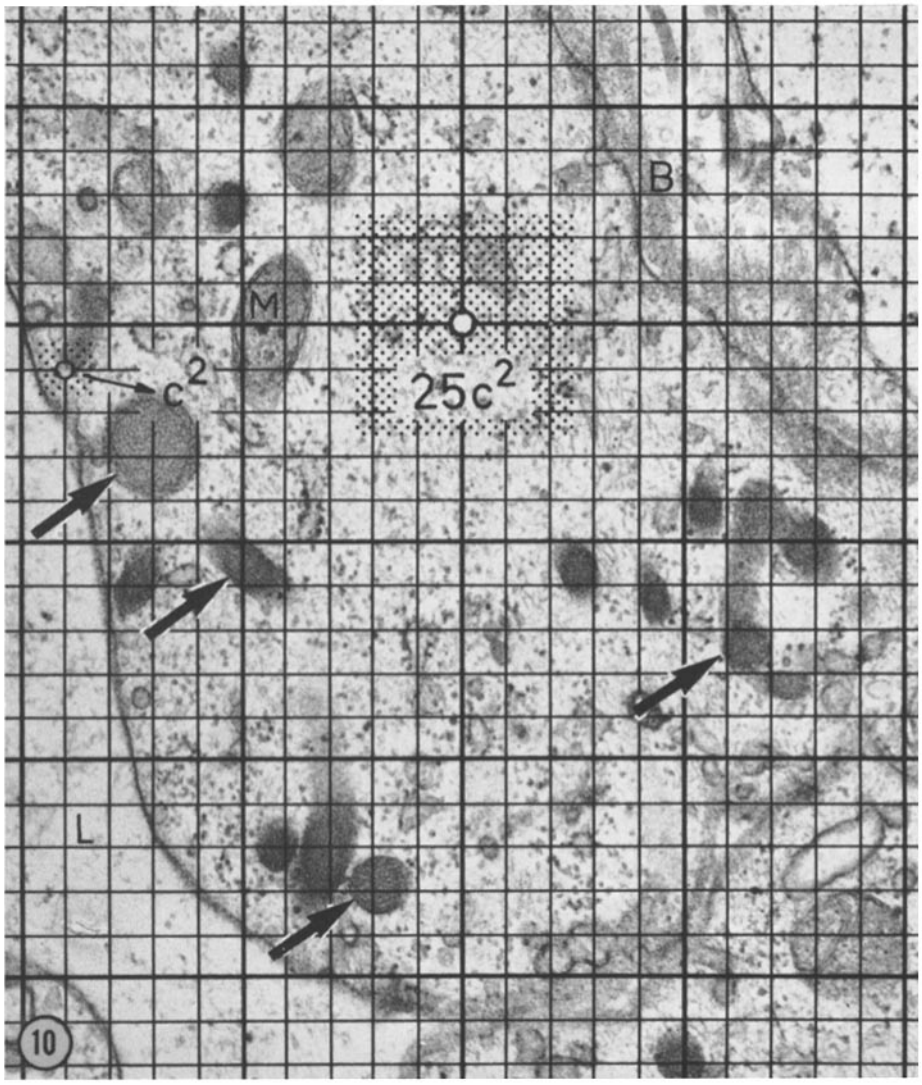


FIGURE 10 Sample field of endothelial cytoplasm from human skin venule superimposed with double-lattice test-system. Arrows identify some profiles of the specific organelle investigated. *L*, vessel lumen, *B*, basement membrane, *M*, mitochondrion. × 44,000.

(P_c') to estimate the area of cytoplasm present on the sample. Then the number of all cross-points lying on organelles (P_k) was recorded. The relative volume V_{Vk} of the organelles in the cytoplasm was obtained from equation 16.

In this last example, section thickness has to be taken into consideration, since the size of the organelles is of about the same dimension. The value of V_{Vk} obtained from the data overestimates the true value by about 50%.

D. CALIBRATION: In order to avoid unnecessary intermediate errors in calibration, it is advisable to record a calibration test specimen (silver screen, carbon-grating replica) on each film strip. At the end of evaluation in the projector unit, the absolute value of the test unit, Z, is determined with this calibration recording. It should be noted that calibration is essentially irrelevant if volumetric work by point-counting alone is performed.

With this procedure, we found very small variations in calibration. These were always due to

variations in the intermediate lens settings and were thus identical for calibration specimen and sections. It is, of course, necessary to observe all precautions for adequate calibration (5, 36).

E. DATA RECORDING: Since the necessary measurements are essentially reduced to a differential *counting* of different "hits" and "cuts," the data can be conveniently recorded in a simple counter unit. Commercially available hematological counters can be used. For more extensive work, we have constructed a more efficient electric counter unit with 10 counters and two totalizers (Fig. 7). The counters are arranged in series with one of the two totalizers and are fed with single impulses from Miltac microswitches which are mounted on two keyboards to allow easy handling with both hands (Fig. 7). At the end of each counting sequence, the counters are read off onto tables.

DISCUSSION

The methods of measurement reviewed in this paper are very efficient and yield reliable results on numerous propositions (44, 35, 30, 46, 15, 40). However, two types of errors may affect this work: (*a*) systematic errors due to the techniques employed, and (*b*) statistical errors. These should be discussed here without entering into details, since they should not lead to serious difficulties if the necessary precautions are observed.

Among the *systematic errors*, tissue-processing artifacts are most important (49). These can be eliminated almost entirely if the fixation and processing media are carefully adjusted to isotonicity. For comparative work, they are further reduced by strict standardization of the laboratory techniques. Under special circumstances, however, the possibility of some residual systematic error has to be borne in mind. If this cannot be eliminated, appropriate mathematical correction should be considered (44).

The principles stated above apply only to sections of (nearly) infinite thinness. Finite section thickness will introduce a certain systematic error (Holmes effect, 29) which depends on the ratio of section thickness to mean "diameter" of the structures investigated. As a general rule, the Holmes effect can be disregarded, if this ratio is much smaller than 1. For example, no problem arises when measuring the volume fraction of whole mitochondria in liver or muscle cells or their external surface-to-volume ratio on ordinary ultrathin sections of silver interference color $\sim$(500 A). However, for determining the surface-to-volume ratio of inner mitochondrial membranes and matrix, the section thickness has to be reduced as far as possible. If two populations of mitochondria have to be compared, the residual Holmes effect can be disregarded if it can be expected to affect both populations to the same degree. Such very thin sections will show little contrast; this can be improved by heavily "over-staining" the sections, for example, with a combined uranyl acetate and lead stain (17). In this work, the aesthetic quality of the electron micrographs is not of primary importance.

The Holmes effect shall not be further treated here. It has received thorough consideration elsewhere for various general cases (29, 23–26, 2, 44).

Statistical Errors

In practical application, the stereological methods proposed here will yield estimates of "true dimensions" within certain confidence intervals which essentially depend on the size of the sample and can be tested by means of usual statistical techniques (43). The quality of the result can be improved by increasing the sample size, but it must be borne in mind that this improvement runs proportional to the square root of the sample size (25, 44).

The distribution of the sample over the tissue is an important factor in reducing the error. Firstly, it has been shown that an even distribution of sample sections in the organ can be achieved by stratified random sampling (44, 13). Hennig (26) has further shown that the use of a systematic lattice of points in the test system, as proposed above, is able to reduce the probable error as compared to a purely random test system. The "randomness" required by the theory of stereology is still preserved if there is no coincidence between test system lattice and any lattice underlying tissue organization. If some structures show preferential orientation, it may be necessary to rotate the test system by, e.g., 90° or two times 60° and take two or three readings (39).

An increase of the sample size can be obtained by three principal steps: (*a*) an increase in the number of sections investigated, (*b*) an increase in the number of micrographs prepared per section, or (*c*) by increasing the point density of the test system. Since we are dealing with multiple-stage sampling (9), the first step will bring the most intense improvement, and the least effect has to

TABLE II

Production of Four-Stage Sample in Experimental Series (30)

Sample stage	1	2	3	4
Characteristics	Specimens	Random sections	Random electron micrographs	Test points analyzed
Personnel required	Investigator + Technician	Technician	Techn. Assist. or Investigator	Investigator
Standard sample per				
Electron micrograph				168
Section			6	1,008
Specimen		5	30	5,040
Experimental group	5	25	150	25,000
Time* required per				
Electron micrograph, min			8	4
Section, min		45	45	24
Specimen, hr		4	4	2
Experiment, hr	(8)‡	20	20	20§
Relative production costs per unit‖	0.2‡	0.5	0.3	<0.001

* Includes all ancillary work.
‡ Preparation time only.
§ Includes statistical analysis.
‖ Wages, material, instrument time, etc.

be expected from simply increasing the test point density. This is immediately seen, if the variance $V(\bar{\bar{y}})$ of the mean obtained by three-stage sampling is considered (9). Taking S_1^2 to be the variance of the first sampling stage, i.e. the variance between sections, and n_1 the corresponding sample size (number of sections); taking furthermore S_2^2 and n_2 to be variance and number of sample fields (electron micrographs) per section, S_3^2 and n_3 variance and number of test points per field, we obtain

$$V(\bar{\bar{y}}) = \frac{S_1^2}{n_1} + \frac{S_2^2}{n_1 . n_2} + \frac{S_3^2}{n_1 . n_2 . n_3} \qquad (17)$$

It is easily seen that an increase in n_1 will reduce all three components while a simple increase in n_3 will only influence the last member, namely S_3^2.

On the other hand, increasing the number of sections will entail the highest increase in the costs of the project. Some estimates of time requirement and effective costs as they pertained to our studies are presented in Table II. Each investigator will have to judge, on the basis of his facilities, which step ought to be increased to provide an optimal result with tolerable cost increase.

In experimental work, a "zero-order" sample has, of course, also to be taken into consideration: the number of test animals used. In general, this number depends on the experimental conditions and does not directly influence the morphometric work. It may often prove advantageous, though, to head directly for group averages without considering the individual animal values. The variance of this group mean is then calculated on the basis of a four-stage sampling procedure according to equation 17 with n_0 and S_0^2 as the number and variance of the animals used, and so forth. The test-points then become the fourth-order sample, and a corresponding fourth term must be added to equation 17. It is then again evident that an increase in the number of experimental animals will best improve the results.

CONCLUSIONS

Stereological methods provide the means of efficiently producing quantitative data on the

internal structure of organs, tissues, and cells. These methods can easily be applied to cytological work at the electron microscope level of resolution. Although particular caution is indicated in avoiding systematic errors which may result from inadequate preparation, section thickness, and so on, the results are generally very reliable. They can be subjected to statistical analysis in order to test for statistical errors. While the derivation of these principles—which was purposely omitted in this presentation—often involves the use of advanced mathematical tools, their practical application requires no special mathematical knowledge. On the basis of simple counting measurements a large variety of dimensions can be inferred.

A combination of these methods with histochemical and radioautographic techniques may allow quantitative evaluation of tissue composition (33).

This work was supported in part by the Schweizerisches Nationalfonds zur Förderung der wissenschaftlichen Forschung; the National Institutes of Health, United States Public Health Service, through grant RF-57; and by the 6570th Aerospace Medical Research Laboratories under contract AF 61 (052)-784 through the European Office of the Aerospace Research (OAR), United States Air Force.

Received for publication 2 December 1965.

REFERENCES

1. ATTARDI, G., Ueber neue, rasch auszuführende Verfahren für Zellmessungen, *Acta Anat.*, 1953, **18**, 177.
2. BACH, G., Ueber die Grössenverteilung von Kugelschnitten in durchsichtigen Schnitten endlicher Dicke, *Z. wissensch. Mikr.*, 1959, **64**, 265.
3. BACH, G., Ueber die Bestimmung von charakteristischen Grössen einer Kugelverteilung aus der Verteilung der Schnittkreise, *Z. wissensch. Mikr.*, 1963, **65**, 285.
4. BACH, G., Zufallsschnitte durch ein Haufwerk von Rotationsellipsoiden mit konstantem Achsenverhältnis, *Z. angew. Math. u. Physik*, 1965, **16**, 224.
5. BAHR, G. F., and ZEITLER, E., The determination of magnification in the electron microscope. II. Means for the determination of magnification, *Lab. Inv.*, 1965, **14**, 880.
6. CHALKLEY, H. W., Methods for the quantitative morphologic analysis of tissues, *J. Nat. Cancer Inst.*, 1943, **4**, 47.
7. CHALKLEY, H. W., CORNFIELD, J., and PARK, H., A method for estimating volume-surface ratios, *Science*, 1949, **110**, 295.
8. CHAYES, F., Petrographic Modal Analysis, New York, Wiley and Sons, 1956.
9. COCHRAN, W. C., Sampling Techniques, New York, Wiley and Sons, 1953.
10. CORNFIELD, J., and CHALKLEY, H. W., A problem in geometric probability, *J. Washington Acad. Sc.*, 1951, **41**, 226.
11. DE HOFF, R. T., and RHINES, F. N., Determination of the number of particles per unit volume from measurements made on random plane sections: the general cylinder and the ellipsoid, *Tr. AIME*, 1961, **221**, 975.
12. DELESSE, M. A., Procédé mécanique pour déterminer la composition des roches, *Compt. rend. Acad. sc.* 1847, **25**, 544.
13. DUNNILL, M. S., Evaluation of a simple method of sampling the lung for quantitative histological analysis, *Thorax*, 1964, **19**, 443.
14. ELIAS, H., and COHEN, T., Geometrical analysis of inclusions in rat liver cells as seen in electronmicrograms, *Z. Zellforschung.*, 1954, **41**, 407.
15. ELIAS, H., HENNIG, A., and ELIAS, P. M., Some methods for the study of kidney structure, *Z. wissensch. Mikr.*, 1961, **65**, 70.
16. ELIAS, H., SOKOL, A., and LAZAROWITZ, A., Contribution to the geometry of sectioning. II. Circular Cylinders, *Z. wissensch. Mikr.*, 1954, **62**, 20.
17. FRASCA, J. M., and PARKS, V. R., A routine technique for double-staining ultrathin sections using uranyl and lead salts, *J. Cell Biol.*, 1965, **25**, 157.
18. FUCHS, A., and WEIBEL, E. R., Morphometrische Untersuchung der Verteilung einer spezifischen cytoplasmatischen Organelle von Endothelzellen in der Ratte, *Z. Zellforschung.*, 1966, **73**, 1–9.
19. GLAGOLEFF, A. A., On the geometrical methods of quantitative mineralogic analysis of rocks, *Tr. Inst. Econ. Min. and Metal. Moskau*, 1933, **59**.
20. HAUG, H., Die Treffermethode, ein Verfahren zur quantitativen Analyse im histologischen Schnitt, *Z. Anat. Entwcklngs. Gesch.*, 1955, **118**, 302.
21. HAUG, H., Quantitative Untersuchungen an der Sehrinde, Stuttgart, Thieme, 1958.
22. HENNIG, A., Bestimmung der Oberfläche beliebig geformter Körper mit besonderer Anwendung auf Körperhaufen im mikroskopischen Bereich, *Mikroskopie*, 1956, **11**, 1.
23. HENNIG, A., Diskussion der Fehler bei der Volumenbestimmung mikroskopisch kleiner Körper

oder Hohlräume aus den Schnittprojektionen, *Z. wissensch. Mikr.*, 1957, **63**, 67.

24. HENNIG, A., Fehler der Oberflächenbestimmung von Kernen bei endlicher Schnittdicke, *Mikroskopie*, 1957, **12**, 7.
25. HENNIG, A., A critical survey of volume and surface measurements in microscopy, *Zeiss Werkzeitschrift*, No. 30, 1959.
26. HENNIG, A., Das Ausmessen räumlicher Strukturen mittels zufälliger Sonden (Punkten, Linien, Ebenen), *in* Proceedings of Symposium on Quantitative Methods in Morphology, (E. R. Weibel and H. Elias, editors), Berlin-Heidelberg-New York, Springer, in press.
27. HENNIG, A., and MEYER-ARENDT, J. R., Microscopic volume determination and probability, *Lab. Inv.*, 1963, **12**, 460.
28. HILLIARD, J. E., and CAHN, J. W., Evaluation of the procedures in quantitative metallography for volume fraction analysis, *Tr. AIME*, 1962, **221**, 344.
29. HOLMES, A. H., Petrographic methods and calculations, London, Murby & Co., 1927.
30. KISTLER, G. S., CALDWELL, P. R. B., and WEIBEL, E. R., A quantitative electron microscope study of lung damages due to breathing of pure oxygen at atmospheric pressure, Proceedings 3rd International Conference on Hyperbaric Medicine, 1965, in press.
31. KNIGHT, B. W., WEIBEL, E. R., and GOMEZ, D. M., Effect of size distribution on a principle of counting on sections structures contained in a volume, Proceedings 1st International Congress on Stereology, Vienna, Vienna Medical Academy, 1963, 18.
32. LAZAROW, A. and CARPENTER, A. M., Component quantitation of tissue sections. I, *Histochem. and Cytochem.*, 1962, **10**, 324 and 329.
33. LEIBNITZ, L., Das Punktzählverfahren als quantitative Methode in der Histochemie, *Histochemie*, 1964, **4**, 123.
34. LOUD, A. V., A method for the quantitative estimation of cytoplasmic structures, *J. Cell Biol.*, 1962, **15**, 481.
35. LOUD, A. V., BARANY, W. C., and PACK, B. A., Quantitative evaluation of cytoplasmic structures in electron micrographs, *Lab. Inv.*, 1965, **14**, 996.
36. REISNER, J. H., The determination of magnification in the electron microscope. I. Instrumental factors influencing the estimate of magnification, *Lab. Inv.*, 1965, **14**, 875.
37. ROSIWAL, A., Ueber geometrische Gesteinsanalysen. Ein einfacher Weg zur ziffermässigen Feststellung des Quantitätsverhältnisses der Mineralbestandteile gemengter Gesteine, *Verhandl. K.K. Geol. Reichsanstalt*, Wien, 1898, 143.
38. SCHUCHARDT, E., Die Gewebsanalyse mit dem Integrationsokular, *Z. wissensch. Mikr.*, 1954, **62**, 9.
39. SITTE, H., Morphometrische Untersuchungen an Zellen, *in* Proceedings of the Symposium on Quantitative Methods in Morphology, (E. R. Weibel and H. Elias, editors), Berlin-Heidelberg-New York, Springer, in press.
40. SITTE, H., and STEINHAUSEN, M., Stereologische Auswertung elektronenoptischer Aufnahmen der Säugerniere, Proceedings 1st International Congress on Stereology, Vienna, 1963, 24.
41. TOMKEIEFF, S. I., Linear intercepts, areas and volumes, *Nature*, 1945, **155**, 24.
42. UNDERWOOD, E. E., Quantitative Stereology, Cambridge, Massachusetts, Addison-Wesley Co., in press.
43. VAN DER WAERDEN, B. L., Mathematische Statistik, Berlin-Heidelberg-New York, Springer, 1965, 2nd edition.
44. WEIBEL, E. R., Morphometry of the human lung, Heidelberg, Springer-Verlag, New York, Academic Press Inc., 1963.
45. WEIBEL, E. R., Principles and methods for the morphometric study of the lung and other organs, *Lab. Inv.*, 1963, **12**, 131.
46. WEIBEL, E. R., Postnatal growth of the lung with respect to gas exchange capacity, *in* Ciba Foundation Symposium on the development of the lung, London, Churchill, in press.
47. WEIBEL, E. R., and ELIAS, H. (editors), Proceedings of Symposium on Quantitative Methods in Morphology, Berlin-Heidelberg-New York, Springer-Verlag, in press.
48. WEIBEL, E. R., and GOMEZ, D. M., A principle for counting tissue structures on random sections, *J. Appl. Physiol.*, 1962, **17**, 343.
49. WEIBEL, E. R., and KNIGHT, B. W., A morphometric study on the thickness of the pulmonary air-blood barrier, *J. Cell Biol.*, 1964, **21**, 367.
50. WEIBEL, E. R., and PALADE, G. E., New cytoplasmic components in arterial endothelia, *J. Cell Biol.*, 1964, **23**, 101.