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# MORPHOMETRY OF MOUSE LUNGS

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BY

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MORPHOMETRY OF MOUSE LUNGS

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# MORPHOMETRY OF MOUSE LUNGS

## CHAPTER I

## INTRODUCTION

### Objective of the Study

The objective of this study is to explore the possibilities of using morphometric techniques to supplement conventional descriptive histopathology in the assay of experimental lung alterations in young white mice.

### Morphometry

Many of the morphometric (morphologic measurement) techniques that biologists are currently using (Naeye and Blanc, 1965; Loud,Barany, and Pack, 1965) are based on principles and procedures developed and used by geologists for quantitative studies of complex rocks (Delesse, 1847; Rosiwal, 1898; Lincoln and Reitz, 1913; Shand, 1916; Glagoleff, 1933; Chayes, 1956). These statistico-geometrical techniques form part of a body of methods collectively called "stereology." They are used in geology, petrology, ceramics and metallurgy (Chayes, 1965) as well as in anatomy (Weibel, 1963a; Elias, Hennig, and Elias, 1962) and pathology (Dunnill, 1962a, 1964, 1965).

Stereology is defined by the International Society for Stereology as "the science of spatially interpreting two-dimensional samples of

three-dimensional structures." This is an apt description of the situation that confronts a geologist looking at the polished surface of a rock, a pathologist examining the cut surface of an organ, or a microscopist viewing a histologic section. In each case, the examiner must make inferences about three-dimensional structures from observations of samples that he can adequately study in only two dimensions (Elias, 1965).

Some of the problems encountered in interpreting shapes of solids from what can be seen in thin slices (plane sections) are illustrated in textbooks of histology (Ham, 1961, chap. 2) and pathology (Hopps, 1964, p. 377). Often overlooked, however, is the fact that certain kinds of reliable quantitative information can be inferred from slices or sections (Eränkö, 1955, p.3). Weibel (1963b) has used the term "morphometry" to designate some methods that can be used for the quantitative morphologic analysis of lungs and other organs. Some of these methods, which permit estimates of (1) relative volumes of the various anatomic components of lungs, (2) the number of alveoli and (3) the internal surface area of lungs are used in this study. These estimates are based on simple measurements obtained by microscopic examination of tissue sections (Chapters II and III).

## Morphometric Studies of Lungs

By using available morphometric techniques and developing some new ones, Weibel and Gomez (1962a, 1962b) and Weibel (1963a, 1963b, 1964) made very extensive quantitative anatomic studies of 5 normal adult human lungs. They found the total number of alveoli (both lungs) to be remarkably constant at about 300 million in each of the individuals

studied. The total air-tissue interface was about 80 square meters. They also measured airways, capillary surface area, and average thickness of air-blood barrier.

Dunnill (1962b), using some of Weibel's techniques in a study of 20 lungs from 10 infants and children, found a steady increase in the number of alveoli from birth (30 million) to 8 years of age (300 million). He concluded that human lungs grow by forming new respiratory units up to the age of 8 years, and after that age, lungs grow by increasing the size of these units.

Quantitative data regarding lung diffusing area (internal surface area) for 26 species of mammals were presented by Tenney and Remmers (1963). They found that the internal surface area of the lung correlated linearly and directly with resting oxygen consumption over the entire range of mammalian size from shrew to whale.

Since they also found that, between species, lung volumes were generally correlated with body size, it followed that two mammals (different species) of comparable size would have comparable lung volumes, but the one with the higher metabolic rate would have increased internal partitioning (more and smaller alveoli) to provide the greater surface area.

Studying the internal surface area of 23 single (right or left) human lungs, Duguid, Young, Cauna, and Lambert (1964) found normal values of 38 to 44 square meters. Values for emphysematous lungs were less than 30 square meters.

Pathologic human lungs were studied morphometrically by Dunnill (1965) also. Hoping to eventually correlate clinical data and ante-

mortem pulmonary function values with post-mortem lung morphology, he reported results of quantitative studies of the pulmonary anatomy and pathology of 21 patients who had died of a variety of chronic lung diseases. He was able to give numerical values for percentage of lung destroyed by emphysema, number and average size of abnormal air spaces, and number and surface area of persisting normal alveoli.

This present study seeks to obtain similar quantitative information about the pulmonary anatomy and pathology of mice.

## Morphology of Mouse Lungs

Although small and relatively simple, the structure of mouse lungs conforms to the basic mammalian pattern (Engel, 1962, chap. 24). As in rats, the left lung consists of a single lobe; the right lung is divided into 4 lobes.

The tracheo-bronchial tree has very few mucous glands, and goblet cells are not very frequent in normal bronchial epithelium. The ciliated cells of the bronchiolar epithelium are interspersed with so-called Clara cells whose granular cytoplasm is rich in mitochondria. The function of these cells is unknown (Rhodin, 1963, p. 86).

The bronchial tree of the mouse lung is actually a bronchiolar tree (Engel, 1962, p. 228) with only 7-9 generations of branchings (Krahl, 1964, p. 254). The human airway, by contrast, has 22-23 generations of branchings (Weibel, 1963, p. 133). The mouse's airways lack cartilage, except near the hilum. Peribronchial and perivascular lymphoid aggregates are usually present (Engel, 1962, p. 238).

According to Krahl (1964, p. 254), the terminal bronchioles of the mouse lung connect directly to alveolar ducts, without intervening

respiratory bronchioles, but Engel (1962, p. 233) mentions that there are two generations of alveolated (respiratory) bronchioles.

The alveolar ducts, whose walls consist of the frames of alveolar entrances, lead to rotunda-like alveolar sacs which bear the terminal alveoli (Krahl, 1964, p. 256).

The mean alveolar diameter for 6 mice examined by Tenney and Remmers (1963) was 46.6 microns.

## CHAPTER II

## PRINCIPLES OF MORPHOMETRY

The fundamental principles that underlie quantitative studies of organs and tissues have been described in detail by Eränkö (1955) and Weibel (1963b, chap. 3). Some of the most pertinent features, including: (1) preparation of the material, (2) sampling of the material, (3) extrapolation from two-dimensional samples to three-dimensional relations, (4) methods of measuring two-dimensional samples, and (5) limitations of morphometric data, are discussed in this chapter.

### Preparation of the Material

Standardization of all steps in the preparative procedure is necessary if different individual specimens are to be compared. Volume changes, usually shrinkage, regularly occur during the fixation and processing of tissue and the preparation of histologic sections (Stowell, 1941). By making appropriate measurements before, during and after the preparative procedure, it is possible to derive correction factors to compensate for these dimensional changes (Weibel, 1963b, p. 44).

### Sampling

In descriptive morphology, it is customary to sample "typical" regions for careful study, but this arbitrary approach may not give an accurate indication of the over-all frequency and distribution of the

structures observed. On the other hand, it is not practical to study all of the samples from a specimen, since an organ as small as the mouse lung yields a thousand or more histologic sections. Therefore, most morphometric techniques make extensive use of statistical procedures and sampling techniques (Weibel, 1963b, p. 10).

The sampling method to be used depends on the distribution of the structures to be measured. For structures that are randomly or homogenously distributed, random sampling is preferred. A systematic sampling plan may be necessary for analyzing non-randomly distributed structures (Weibel, 1963b, p. 10).

Since anatomic structures often possess a high degree of organization, it is necessary to make some "working rules" regarding randomness. Weibel (1963b, p. 11) suggests that randomness of structures in space may be assumed if the units under investigation do not exhibit any stratified array in the unit tissue volume, even though they may be well organized into units of higher order. In other words, sections in all different planes should yield very similar pictures. Randomness of distribution should refer only to a well specified part of the tissue. Glomeruli, for example, are randomly distributed only with respect to the renal cortex.

## Two-Dimensional Samples

Studies of the microscopic structure of organs are usually carried out on very thin sections, typically less than 10 microns for light microscopy and less than 0.1 microns for electron microscopy. For qualitative and descriptive analyses, it has been intuitively assumed that these slices (histopathologic sections) are representative samples of the larger specimens.

But, can these thin sections, which for practical purposes are two-dimensional samples, be quantitatively representative of the volumetric composition of the original three-dimensional structure?

An affirmative answer was given by Delesse (1847) to a similar problem involving the composition of rocks. The principle that he developed states that if a section is placed through a volume containing a given component, the fraction of the section area covered by transections of the component will be equal to the fraction of the volume occupied by that component.

Formal demonstrations of this principle of the equivalence of areal and volume proportions have been undertaken by Chayes (1965) and Weibel (1963b, p. 12). The following demonstration is patterned after the one given by Weibel (1963b, p. 12).

Suppose that a cube with a volume

$$V = L^3$$

contains granules of any size or shape which together have a volume

$$v = PV = PL^3$$

where P is the proportion of the volume that the granules occupy. Consider a thin slice of thickness dx (parallel to one of the faces of the cube), having a volume

$$dV = L^2 \cdot dx.$$

In this slice, a volume

$$dv = n(x) \cdot dV = n(x) \cdot L^2 \cdot dx$$

will contain sections of the granules. L and dx are constant wherever the slice is placed, but n(x) will vary with the placement of the slice. If $dx \rightarrow 0$, the slice becomes very thin and finally reduces to a plane.

The total volume of the granules, v, is then

$$v = \int_0^L dv = L^2 \cdot \int_0^L n(x) \cdot dx = PL^3$$

where n(x) is the fraction of the intersecting plane covered by transections of the component at any position x.

Since

$$\frac{1}{L} \cdot \int_0^L n(x) \cdot dx = \bar{n}$$

is the average value of the coefficient n(x) between 0 and L, it follows that

$$L^2 \cdot \int_0^L n(x) \cdot dx = \bar{n} L^3 = PL^3$$

and therefore

$$\bar{n} = P.$$

This means that an average fractional coefficient ($\bar{n}$) representing the proportion of area occupied by a component on sections through a three-dimensional structure, represents an estimator of the volumetric proportion (P) of the component under investigation.

## Methods of Measurement

### Volumetric Proportions of Anatomic Components

Acceptance of Delesse's principle reduces the determination of relative volumes to the measurement of relative areas on sections. This can be accomplished by (1) planimetry of photographs, tracings, or projected images, (2) relative weights of components of paper or foil tracings or of photographs, (3) line sampling, and (4) point

sampling (Eränkö, 1955, chap. 6). The complexity of the internal structure of the lung practically precludes the use of the first two methods.

The linear integration (line sampling) method. Extending the principle of Delesse (1847), the geologist Rosiwal (1898) demonstrated that the fraction of a line passing through a randomly distributed component is approximately equal to the fraction of the volume occupied by this component. This led to the development of integrating mechanical stages (Shand, 1916) and integrating eyepieces (Schuchardt, 1954), for microscopes. Mechanical scanning and recording devices have been devised also (Eränkö, 1955 p. 82; Lazarow and Carpenter, 1962).

The point counting method. The principles of Delesse (1847) and Rosiwal (1898) can be further extended to a procedure of point counting. If a given proportion of counting points overlie one particular kind of tissue, then that tissue occupies the same proportion of space within the whole mass of the organ under consideration (Elias, 1965). This principle, introduced by the geologist Glagoleff (1933), was first used on biological tissues by Chalkley (1943). The technique was used by Dunnill (1962a) for quantitative studies of human pulmonary pathology.

## Number of Alveoli

Weibel and Gomez (1962a) developed a principle which permits direct estimation of the number of structures contained in a volume of tissue from simple counts on histologic sections. They showed that the relation depends on the volumetric density and the shape of the structures. If a unit volume contains N granules of equal size and

shape and n transections (slices) of these granules appear on the unit area of the cut surface, then

$$N = \frac{n^{3/2}}{B \cdot P^{\frac{1}{2}}}$$

where P is the volumetric density as determined by Delesse's principle and B is a constant relating to shape. The value of B for alveoli is given as 1.55.

A subsequent publication (Weibel, 1963b, p. 26) described a distribution coefficient which corrected for differences in size of the structures. Because the variation in alveolar diameters was assumed to be relatively small, this coefficient was neglected in practice (Weibel, 1963b, p. 28).

## Surface Area

The internal surface area of a complex, partitioned, three-dimensional structure, such as a lung, can be determined by the method of Campbell and Tomkeieff (1952). If a line of known length (L) is placed randomly on a random section of lung it will intercept alveolar septa m times. If this is done a sufficient number (N) of times, then

$$L_m = \frac{N \cdot L}{\sum m}$$

will represent the "mean chord length" of the randomly placed lines with alveolar membranes. It has been shown that the overall surface area ($S_A$) of the double-faced alveolar membrane follows from the equation

$$S_A = \frac{4 \cdot V_L}{L_m}$$

where $V_L$ is the volume of the respiratory portion of the lung (Weibel, 1963b, p. 37).

Because of corrugations of the alveolar surfaces, the actual air-tissue interface may be about 20% greater than the surface area determined by this method (Weibel, 1963b, p. 70).

## Limitations of Morphometric Data

It should be recognized that there are many uncertainties in quantitative studies of morphology. Many assumptions and approximations have to be made. In this kind of work the verb "to estimate" is probably more appropriate than "to measure" (Chayes, 1965). Yet, these data can still be useful, if the limitations are kept in mind when the interpretations are made (Eränkö, 1955, p. 8).

The extraction of numerical data from a single histologic section is a relatively simple task, and by making enough microscopic measurements a high degree of accuracy and precision can be achieved. The value of such data from a single section is limited, however, unless information is available regarding the preparative and sampling procedures that produced that particular section for morphometric analysis.

# CHAPTER III

# MATERIALS AND METHODS

## Materials

### Animals

All animals used in this study were Caeserean-originated, barrier-sustained, "COBS", white mice obtained at 4-5 weeks of age from Charles River Mouse Farms, Inc., North Wilmington, Massachusetts (Foster, Foster, and Pfau, 1963). They were housed in "clean" facilities, away from other rodents, in an effort to keep them free from the spontaneous respiratory infections that are prevalent among laboratory rodent colonies (Innes, 1965).

Twenty four female mice between 4 and 14 weeks of age were studied. Their weights (at autopsy) ranged from 19.3 to 42.1 grams (Table 1).

Six of the mice were treated experimentally; 18 were untreated controls.

Two of the experimental mice (7a, 8a) were given droplets of a broth culture of a strain of streptococci known to be pathogenic for mice, by nasal insufflation. One mouse (7a) was sacrificed 48 hours after receiving the bacteria; the other mouse (8a) was sacrificed 72 hours after receiving the bacteria.

TABLE 1

CONTROL AND EXPERIMENTAL PAIRS

| Treatment | Animal # | Age Wks. | Sex | Body Wt.[a] (gm.) | Lung Volume | | Conversion Factor[b] | | | Approx. No. of 5μ Serial Sections | Lung Wt. (gm.) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Std. ($cm^3$) | Processed | Vol. | Area | Linear | | |
| **Control Pairs** | | | | | | | | | | | |
| Control | 1a | 4 | F | 19.5 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 975 | 0.195 |
| Control | 1b | 4 | F | 19.3 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 975 | 0.163 |
| Control | 2a | 5 | F | 21.7 | 1.0 | 0.25 | 4.00 | 2.53 | 1.59 | 975 | 0.194 |
| Control | 2b | 5 | F | 21.1 | 1.0 | 0.25 | 4.00 | 2.53 | 1.59 | 875 | 0.186 |
| Control | 3a | 5 | F | 21.9 | 1.0 | 0.25 | 4.00 | 2.53 | 1.59 | 1000 | 0.182 |
| Control | 3b | 5 | F | 22.4 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 875 | 0.190 |
| Control | 4a | 8 | F | 29.8 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 1050 | 0.210 |
| Control | 4b | 8 | F | 29.8 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 1075 | 0.180 |
| Control | 5a | 10 | F | 31.0 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 975 | 0.250 |
| Control | 5b | 10 | F | 30.3 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 900 | 0.225 |
| Control | 6a | 14 | F | 41.3 | 1.0 | 0.34 | 2.94 | 2.05 | 1.43 | 1150 | 0.228 |
| Control | 6b | 14 | F | 35.4 | 1.0 | 0.33 | 3.00 | 2.09 | 1.44 | 1075 | 0.196 |
| **Experimental Pairs** | | | | | | | | | | | |
| Strep. | 7a | 6 | F | 25.2 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 1700 | .. |
| Control | 7b | 6 | F | 25.6 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 1125 | .. |
| Strep. | 8a | 6 | F | 23.5 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 1375 | .. |
| Control | 8b | 6 | F | 25.7 | 1.0 | 0.3 | 3.33 | 2.22 | 1.49 | 1050 | .. |
| $SO_2$ | 9a | 14 | F | 22.3 | 1.0 | 0.27 | 3.70 | 2.39 | 1.55 | 1000 | 0.198 |
| Control | 9b | 14 | F | 41.8 | 1.0 | 0.26 | 3.85 | 2.46 | 1.57 | 1000 | 0.240 |
| $SO_2$ | 10a | 14 | F | 32.7 | 1.0 | 0.28 | 3.57 | 2.33 | 1.53 | 1075 | 0.232 |
| Control | 10b | 14 | F | 38.9 | 1.0 | 0.26 | 3.85 | 2.46 | 1.57 | 1000 | 0.232 |
| $NO_2$ | 11a | 14 | F | 36.3 | 1.0 | 0.31 | 3.23 | 2.18 | 1.48 | 1000 | 0.331 |
| Control | 11b | 14 | F | 37.5 | 1.0 | 0.28 | 3.57 | 2.33 | 1.53 | 1200 | 0.230 |
| $NO_2$ | 12a | 14 | F | 31.3 | 1.0 | 0.34 | 2.94 | 2.05 | 1.43 | 1250 | 0.307 |
| Control | 12b | 14 | F | 42.1 | 1.0 | 0.33 | 3.00 | 2.09 | 1.44 | 1000 | 0.221 |

[a] Body weight at autopsy.

[b] Factor for converting processed to standard dimensions.

Two experimental mice (9a, 10a) were exposed for 8 days to an atmosphere containing sulfur dioxide ($SO_2$), about 100 parts per million, in a controlled atmosphere chamber by the Pharmacology and Toxicology Section, Laboratory of Medical and Biological Sciences, Division of Air Pollution, United States Public Health Service at the Air Pollution Research Facility of the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

Two experimental mice (11a, 12a) were exposed for 8 days to an atmosphere containing oxides of nitrogen ($NO_2$), 15-20 parts per million, in a comparable exposure chamber.

## Paired Animals

Since the mice were not all the same age or same size, it was decided to study the differences between paired animals. Pairings were made at the beginning of the experiment. The 2 members of each pair were matched in respect to age, sex, weight, and general appearance. Since litter mates were not available, the pair mates were always selected from the same shipment from the animal supplier.

By lot, one member of each pair was designated "a", the other was designated "b". For each of 12 morphometric measurements, the value for "b" was subtracted from "a". These differences formed the basis for the statistical analysis.

Six of the pairs were "Control Pairs", that is, neither member received an experimental treatment.

Six pairs were "Experimental Pairs", in which animal "a" was an experimental animal, and its mate, animal "b" was an untreated control.

## Methods

### Preparation of the Specimens

The animals were sacrificed by injecting an overdose (about 25 milligrams) of pentobarbital into the peritoneal cavity. They were promptly autopsied and the thoracic viscera were carefully dissected. After separating the lungs from the heart, thymus, esophagus, and mediastinal lymph nodes, the trachea was cannulated with a 24 gauge hypodermic needle. The tracheo-bronchial tree was infused with 10% bufferred formalin from a reservoir whose fluid level was held about 28-30 centimeters above the level of the lung. This amount of hydrostatic pressure did not distort the lung architecture. When, by visual inspection, the lung contours appeared full and the lung fissures were approximated, the cannula was removed and the trachea was ligated. This"inflation" procedure, which required only 5-10 minutes, was the simplest and also the most satisfactory of several methods that were tried. Other inflation-fixation techniques are recommended for larger lungs (Weibel and Vidone, 1961).

The formalin-filled lungs were immersed in 10% buffered formalin for 48 hours or longer before being dehydrated, cleared, and infiltrated with paraffin in an automatic tissue processing unit. The paraffin-embedded lungs were mounted on a rotary microtome and serial sections, 5 microns in thickness, were cut in a transverse plane, beginning at the diaphragmatic surface and progressing toward the apex. This procedure produced about 1,000 sections from each of the lungs, except numbers 7a and 8a which had been set at a different angle in the paraffin block (Table 1).

Sets of at least 10 numbers (plus 5-10 spares) between 1 and 1,000, drawn from a random number table, designated the sections from each specimen to be mounted on glass slides and stained with hematoxylin and eosin. This gave a set of at least 10 random histologic sections ("two-dimensional samples") from each lung for the morphometric studies.

## Measurement of Lung Volumes and Calculation of Shrinkage Correction Factors

There were three values for lung volume to be considered. The first, a "fresh" or "living" volume, was that which existed during life. Since this volume changed constantly during the breathing cycle, a single value could not be defined, except in an arbitrary way. It was decided to follow the suggestion of Duguid, Young, Cauna, and Lambert (1964) who, in a study of human lungs, assigned to each specimen an arbitrary "standard" lung volume of 3,000 $cm.^3$ After measuring several mouse lungs at various degrees of inflation it was decided to arbitrarily designate 1.0 $cm.^3$ as the "standard" lung volume for the mice in this study. This convenient value was chosen after finding that the deflated lungs always measured less than 1.0 $cm.^3$ and the fully inflated lungs always measured more than 1.0 $cm.^3$

The "processed" volume was the third lung volume to be considered. This volume tried to define the size of the shrunken specimen on which the microscopic measurements were actually made. It was measured by water displacement in a graduated cylinder after the lung had been infiltrated with paraffin, but before it was embedded in the larger paraffin block for sectioning. In an attempt to get readings to the nearest 0.01 $cm.^3$, the cylinder was calibrated and read through a cathetometer.

Processed lung volumes and correction factors for each lung are listed in Table 1. The shrinkage correction factor for volume was obtained by dividing the standard lung volume by the processed lung volume. The correction factor for linear dimensions was the cube root of the correction factor for volume. The correction factor for area was the square of the correction factor for linear dimensions (or the square of the cube root of the correction factor for volume) (Weibel 1963b, p. 45).

No allowances were made for dimensional changes that might have occurred during sectioning, mounting, and staining. After making a few measurements, it was decided that these changes did not warrant the introduction of an additional correction factor. Admittedly, this decision was influenced by the additional time required for making these measurements during the already lengthy serial sectioning procedure.

## Measurements and Calculations

Estimation of volumetric proportions. Volumetric proportions of the various anatomic components of the lungs were estimated by the point counting method (Chapter II). For each animal, the set of random sections was examined under low magnification (8x eyepiece, 10x objective).

A sampling pattern was developed to facilitate orderly examination of a large portion of the area of the section without skipping parts or examining them more than once. Microscopic fields, selected according to the pattern illustrated in Figure 1, numbered about 100 per specimen, with a range from 70 to 215 (Table 2). The microscope's

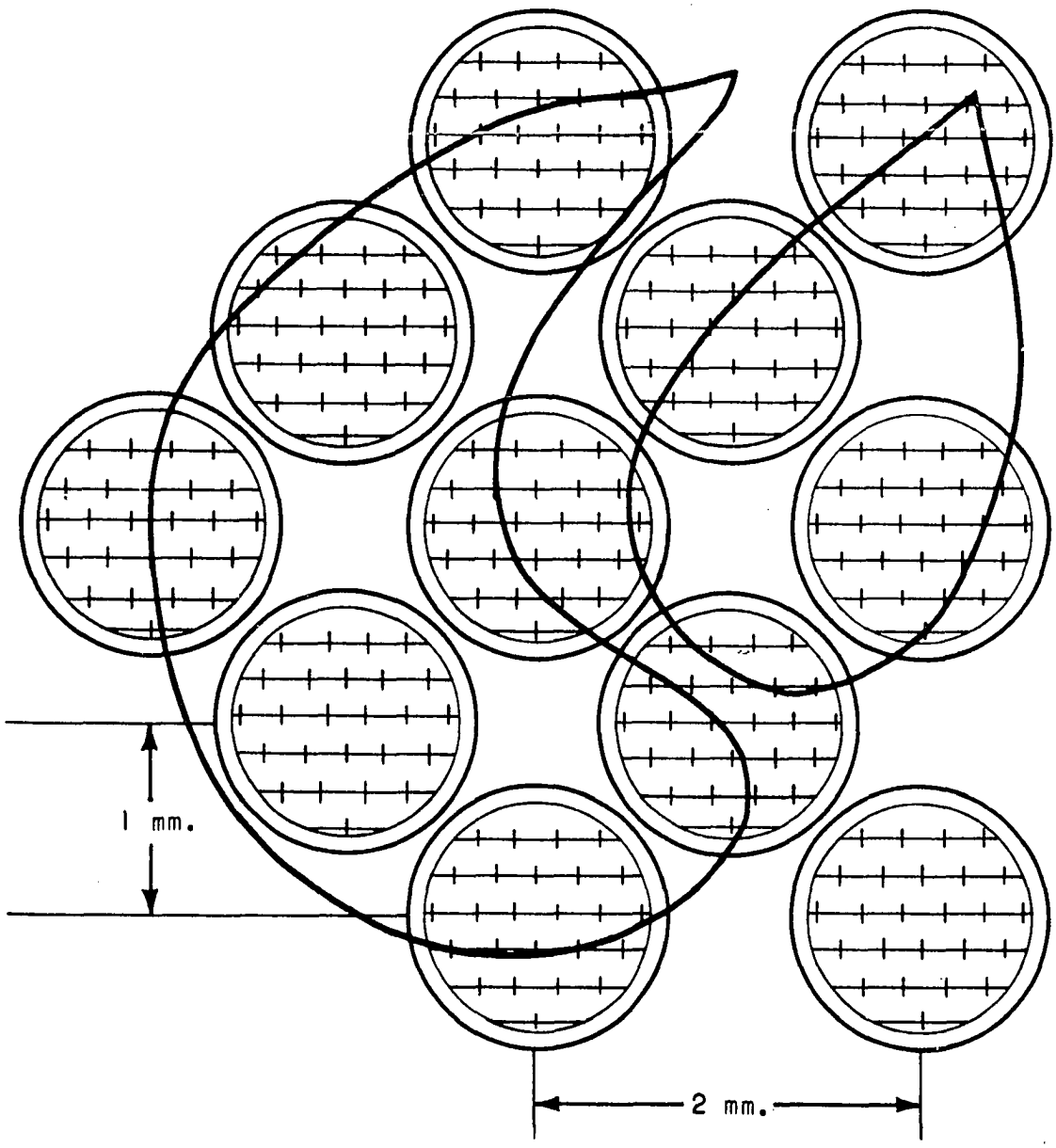


**Figure 1. - Pattern for selecting microscopic fields, superimposed over profiles of tissue sections.**

TABLE 2

DIFFERENTIAL POINT COUNTS FOR ESTIMATION OF VOLUMETRIC PROPORTIONS

| Animal Number | Number of Points Counted | | | | | | Number of Microscopic Fields Examined |
|---|---|---|---|---|---|---|---|
| | A Alveolar Air | D Alveolar Duct Air | S Alveolar Septa | C Bronchi, Blood Vessels | I Inflam.& Lymphoid Tissue | T Total Points Counted | |
| **Control Animals** | | | | | | | |
| 1a | 1546 | 729 | 555 | 453 | 31 | 3314 | 119 |
| 1b | 1521 | 676 | 519 | 553 | 0 | 3269 | 96 |
| 2a | 1835 | 974 | 618 | 632 | 0 | 4059 | 120 |
| 2b | 1279 | 631 | 415 | 415 | 0 | 2792 | 87 |
| 3a | 1864 | 808 | 553 | 640 | 120 | 3985 | 136 |
| 3b | 1689 | 739 | 482 | 494 | 0 | 3404 | 107 |
| 4a | 1268 | 594 | 477 | 498 | 0 | 2837 | 111 |
| 4b | 1303 | 530 | 381 | 455 | 8 | 2677 | 107 |
| 5a | 1357 | 560 | 467 | 449 | 4 | 2837 | 107 |
| 5b | 1269 | 528 | 441 | 422 | 5 | 2665 | 101 |
| 6a | 1158 | 543 | 453 | 469 | 0 | 2623 | 101 |
| 6b | 1935 | 807 | 743 | 650 | 8 | 4143 | 165 |
| 7b | 1133 | 592 | 510 | 421 | 0 | 2656 | 79 |
| 8b | 1586 | 686 | 678 | 484 | 0 | 3434 | 96 |
| 9b | 1250 | 488 | 428 | 542 | 0 | 2708 | 106 |
| 10b | 1537 | 608 | 539 | 512 | 0 | 3196 | 126 |
| 11b | 1638 | 645 | 569 | 514 | 0 | 3366 | 134 |
| 12b | 1664 | 681 | 613 | 585 | 0 | 3543 | 141 |
| **Experimental Animals** | | | | | | | |
| 7a | 1017 | 398 | 460 | 482 | 98 | 2455 | 113 |
| 8a | 1676 | 1087 | 1061 | 1102 | 2073 | 6999 | 152 |
| 9a | 1149 | 510 | 652 | 480 | 0 | 2791 | 109 |
| 10a | 1188 | 551 | 612 | 561 | 0 | 2912 | 114 |
| 11a | 2114 | 1061 | 1243 | 934 | 40 | 5392 | 215 |
| 12a | 1763 | 915 | 1020 | 593 | 19 | 4310 | 170 |

graduated mechanical stage was set at an integer on the "vertical" scale. The slide was traversed along the "horizontal" scale until the tissue section came into view and then a microscopic field was examined at every second whole-numbered position. When the tissue had been traversed in this manner, the stage was set at the next mark (1 mm.) on the vertical scale and the horizontal traverse was repeated. To make the pattern easy to use, microscopic fields were examined at each even numbered mark (2 mm. apart) on the horizontal scale when the vertical scale was set at an even number. On the next traverse, with the vertical scale at an odd number, fields were examined at the odd numbered marks of the horizontal scale. The relation of the tissue section to this sampling pattern was assumed to be random, since it was determined by the position and orientation that the section assumed when it was floated onto the glass slide by the histologic technician.

A Zeiss Integrating Eyepiece I, with 25 points in a hexagonal lattice (Figure 2), provided the points for the differential point counting analysis. In each microscopic field the points were optically superimposed on the magnified image of the specimen. They were registered on a manually operated mechanical tally counter according to the type of anatomic structure that they "hit."

The number of categories for anatomic components of normal lung was limited to 5 in order to permit the examiner to make the tallies with the fingers of one hand, on one counter, without shifting his eyes from the microscopic field. These categories were: alveolar air (A), alveolar duct and sac air (D), alveolar septa (S), connecting

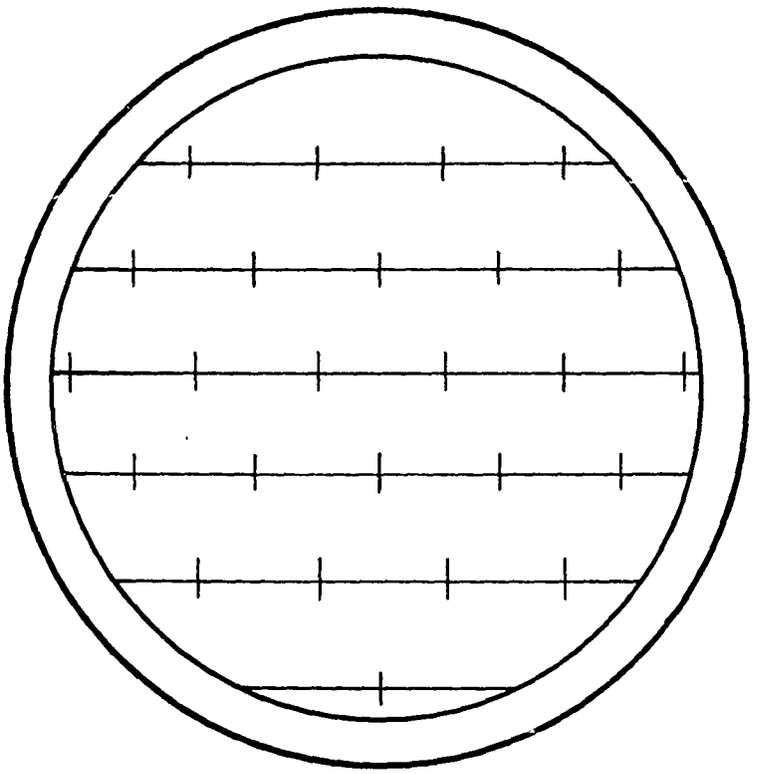

Figure 2. - Reticle for differential point counting (Zeiss Integrating Eyepiece I).

and conducting structures, such as bronchi, bronchioles, blood vessels, and connective tissues (C), and inflammatory and lymphoid tissues (I). This type of analysis required that the categories be all inclusive and mutually exclusive, that is, every point should meet the criteria for one, but only one, category. The criteria should be distinct enough that little subjective judgment be needed to make the differential tallies.

Table 2 contains a summary of the differential point counts for each of the 24 animals. Complete data sheets for one animal, showing the point counts for each microscopic field, are included in Appendix I.

For each field, a proportion ($p_i$) for any category could be calculated by the formula

$$p_i = \frac{a_i}{m_i}$$

where $a_i$ is the tally for the category of interest and $m_i$ is the total number of points counted in that field.

A decision had to be made regarding microscopic fields that were not completely filled by the image of the specimen. To reject them and count only "full" fields (25 points) would have reduced the number of suitable fields by more than half. On the other hand, it was felt that analysis of less than 20 points per field would not give adequate estimates for the proportions. Therefore, the following scheme was devised. Fields with less than 10 points overlying the tissue image were not recorded. Fields with 10 to 19 points overlying the tissue were tallied and the eyepiece was rotated $180^{\circ}$ to produce an additional set of points whose counts were included in the record for that field.

Fields with 20 to 25 points overlying tissue were simply tallied and recorded. With this scheme, the number of points per field ranged from 20 to 40 with an average of about 25.

For the entire set of slides from each animal, an overall proportion (p) for any category could be calculated by the formula

$$p = \frac{\sum a_i}{\sum m_i}$$

where each sum was taken over the n microscopic fields.

Since the points in fields represented "cluster sampling," the standard deviation (s) of the $p_i$'s was estimated by the formula

$$s = \sqrt{\frac{1}{(n-1)}\sum\left\{\left(\frac{m_i}{\bar{m}}\right)^2 \left(p_i - p\right)^2\right\}} = \frac{1}{\bar{m}} \sqrt{\frac{1}{(n-1)}\left\{\sum a_i^2 - 2p \sum a_i m_i + p^2 \sum m_i^2\right\}}$$

where

$$\bar{m} = \frac{\sum m_i}{n}$$

is the average size of cluster (points per field) in the sample (Cochran, 1956, p. 500). The standard error ($s_p$) was calculated by the formula

$$s_p = \frac{s}{\sqrt{n}}$$

The coefficient of variation (C.V.) was calculated by the formula

$$C.V. = \frac{s}{p} \times 100\%$$

Some of these calculations are presented in Table 3.

Ten proportions, or fractions, were calculated for each specimen. The first set (Table 4) expressed ratios of the points tallied for each of the 5 categories to the total number of points counted in that lung. An additional fraction had for its numerator the sum (A+D+S) of the

TABLE 3

ESTIMATES OF MAJOR COMPONENTS
OF MOUSE LUNGS

| Animal Number | Alveoli as a Fraction of Parenchyma | | | | Parenchyma as a Fraction of Whole Lung | | | |
|---|---|---|---|---|---|---|---|---|
| | $\frac{(A+S)}{(A+D+S)}$ | Std. Dev. | Coeff. of Var. | Std. Error | $\frac{(A+D+S)}{T}$ | Std. Dev. | Coeff. of Var. | Std. Error |
| **Control Animals** | | | | | | | | |
| 1a | 0.7424 | 0.0817 | 11.00% | 0.00749 | 0.8540 | 0.1162 | 13.61% | 0.0107 |
| 1b | .7511 | .0578 | 7.70 | .00591 | .8308 | .1055 | 12.70 | .0108 |
| 2a | .7158 | .0744 | 10.39 | .00679 | .8443 | .1104 | 13.07 | .0101 |
| 2b | .7286 | .0708 | 9.72 | .00759 | .8327 | .1441 | 17.21 | .0155 |
| 3a | .7495 | .0770 | 10.27 | .00660 | .8093 | .1396 | 17.25 | .0120 |
| 3b | .7460 | .0703 | 9.43 | .00680 | .8549 | .1084 | 12.68 | .0105 |
| 4a | .7460 | .0913 | 12.24 | .00866 | .8245 | .1604 | 19.45 | .0152 |
| 4b | .7606 | .0855 | 11.24 | .00827 | .8270 | .1259 | 15.22 | .0122 |
| 5a | .7651 | .0868 | 11.34 | .00839 | .8430 | .1545 | 18.39 | .0149 |
| 5b | .7641 | .0778 | 10.18 | .00778 | .8398 | .1452 | 17.29 | .0145 |
| 6a | .7479 | .0813 | 10.87 | .00813 | .8212 | .1324 | 16.12 | .0132 |
| 6b | .7684 | .0830 | 10.80 | .00646 | .8412 | .1337 | 15.89 | .0104 |
| 7b | .7351 | .0704 | 9.58 | .00792 | .8415 | .1341 | 15.94 | .0151 |
| 8b | .7675 | .0537 | 7.00 | .00548 | .8591 | .1146 | 13.34 | .0117 |
| 9b | .7747 | .0859 | 11.09 | .00834 | .7999 | .1496 | 18.70 | .0145 |
| 10b | .7735 | .0774 | 10.00 | .00692 | .8398 | .1320 | 15.72 | .0118 |
| 11b | .7738 | .0761 | 9.83 | .00657 | .8473 | .1449 | 17.10 | .0125 |
| 12b | .7698 | .0747 | 9.70 | .00631 | .8349 | .1396 | 16.92 | .0118 |
| **Experimental Animals** | | | | | | | | |
| 7a | 0.7877 | 0.0819 | 10.40% | 0.00764 | 0.7637 | 0.1717 | 22.48% | 0.0162 |
| 8a | .7157 | .0905 | 12.64 | .00734 | .5464 | .2660 | 48.77 | .0216 |
| 9a | .7793 | .0819 | 10.51 | .00785 | .8280 | .1295 | 15.64 | .0124 |
| 10a | .7656 | .0730 | 9.53 | .00683 | .8073 | .1476 | 18.28 | .0138 |
| 11a | .7598 | .0801 | 10.54 | .00546 | .8194 | .1453 | 17.73 | .0099 |
| 12a | .7526 | .0747 | 9.94 | .00573 | .8580 | .1244 | 14.49 | .0095 |

TABLE 4

LUNG COMPONENTS AS A FRACTION OF WHOLE LUNG

| Treatment | Animal Number | $\frac{A}{T}$ Alveolar Air | $\frac{D}{T}$ Alveolar Duct Air | $\frac{S}{T}$ Alveolar Septa | $\frac{C}{T}$ Bronchi Blood Vessels, etc. | $\frac{I}{T}$ Inflam. and Lymphoid Tissue | $\frac{A+D+S}{T}$ Parenchyma |
|---|---|---|---|---|---|---|---|
| | | | Control Animals | | | | |
| - | 1a | 0.4665 | 0.2199 | 0.1675 | 0.1367 | 0.0094 | 0.8540 |
| - | 1b | .4653 | .2068 | .1588 | .1692 | .0000 | .8308 |
| - | 2a | .4521 | .2400 | .1523 | .1557 | .0000 | .8443 |
| - | 2b | .4581 | .2260 | .1486 | .1673 | .0000 | .8327 |
| - | 3a | .4678 | .2027 | .1387 | .1606 | .0301 | .8093 |
| - | 3b | .4962 | .2171 | .1416 | .1451 | .0000 | .8549 |
| - | 4a | .4470 | .2094 | .1681 | .1755 | .0000 | .8245 |
| - | 4b | .4867 | .1980 | .1423 | .1700 | .0030 | .8270 |
| - | 5a | .4783 | .1974 | .1646 | .1583 | .0014 | .8403 |
| - | 5b | .4762 | .1981 | .1655 | .1583 | .0019 | .8398 |
| - | 6a | .4415 | .2070 | .1727 | .1788 | .0000 | .8212 |
| - | 6b | .4671 | .1948 | .1793 | .1569 | .0019 | .8412 |
| - | 7b | .4266 | .2229 | .1920 | .1585 | .0000 | .8415 |
| - | 8b | .4619 | .1998 | .1974 | .1409 | .0000 | .8591 |
| - | 9b | .4616 | .1802 | .1581 | .2001 | .0000 | .7999 |
| - | 10b | .4809 | .1902 | .1686 | .1602 | .0000 | .8398 |
| - | 11b | .4866 | .1916 | .1690 | .1527 | .0000 | .8473 |
| - | 12b | .4697 | .1922 | .1730 | .1651 | .0000 | .8349 |
| Mean | | 0.4661 | 0.2052 | 0.1643 | 0.1617 | 0.0027 | 0.8357 |
| Std. dev. | | .0172 | .0150 | .0161 | .0146 | .0072 | .0153 |
| | | | Experimental Animals | | | | |
| Strep | 7a | 0.4143 | 0.1621 | 0.1874 | 0.1963 | 0.0399 | 0.7637 |
| Strep | 8a | .2395 | .1553 | .1516 | .1575 | .2962 | .5464 |
| $SO_2$ | 9a | .4117 | .1827 | .2336 | .1720 | .0000 | .8280 |
| $SO_2$ | 10a | .4080 | .1892 | .2102 | .1927 | .0000 | .8073 |
| $NO_2$ | 11a | .3921 | .1968 | .2306 | .1723 | .0074 | .8194 |
| $NO_2$ | 12a | .4090 | .2123 | .2367 | .1376 | .0044 | .8580 |

points counted for alveolar air (A), alveolar duct air (D), and alveolar septa (S). Taken together, these 3 categories were designated the "parenchyma" or the respiratory portion of the lung.

The second set of fractions (Table 5) expressed the ratios of alveolar air (A), alveolar duct air (D), alveolar septa (S), and alveoli (A+S) to the lung parenchyma (A+D+S).

These 10 fractions were considered to be estimates of the volumetric proportions of the various anatomic components in the processed lung specimens (Chapter II). To interpret them as estimates of the proportions of the "fresh" or "standard" volumes would require an assumption that shrinkage affected all of the components equally.

Estimation of number of alveoli per mouse. The number of pulmonary alveoli for each mouse was estimated by the method of Weibel and Gomez (1962a). The number (N) of alveoli per unit volume of lung parenchyma is related to the number (n) of alveolar transections per unit area on a section and the volumetric density (P) of alveoli by the equation

$$N = \frac{n^{3/2}}{B \cdot \sqrt{P}}$$

where B equals 1.55 for the alveolar configuration (Chapter II). The number ($N_m$) of alveoli per mouse was obtained by the formula

$$N_m = N \cdot V'$$

where $V'$ is the "processed" volume of the lung parenchyma.

The number (n) of transected alveoli per unit area was counted on Polaroid low magnification (10x eyepiece, 10x objective) photomicrographs according to the following scheme. From the set of random sections, 4 were chosen for photography. Each of 8 photomicrographs

TABLE 5

LUNG COMPONENTS AS A FRACTION OF LUNG PARENCHYMA

| Treatment of Animal | Animal Number | A / A+D+S Alveolar Air | D / A+D+S Alveolar Duct Air | S / A+D+S Alveolar Septa | A+S / A+D+S Alveoli (Air + Septa) |
|---|---|---|---|---|---|
| | | Control Animals | | | |
| - | 1a | 0.5463 | 0.2576 | 0.1961 | 0.7424 |
| - | 1b | .5600 | .2489 | .1911 | .7511 |
| - | 2a | .5355 | .2842 | .1803 | .7158 |
| - | 2b | .5501 | .2714 | .1785 | .7286 |
| - | 3a | .5780 | .2505 | .1715 | .7495 |
| - | 3b | .5804 | .2540 | .1656 | .7460 |
| - | 4a | .5421 | .2540 | .2039 | .7460 |
| - | 4b | .5885 | .2394 | .1721 | .7606 |
| - | 5a | .5692 | .2349 | .1959 | .7651 |
| - | 5b | .5670 | .2359 | .1971 | .7641 |
| - | 6a | .5376 | .2521 | .2103 | .7479 |
| - | 6b | .5552 | .2316 | .2132 | .7684 |
| - | 7b | .5069 | .2649 | .2282 | .7351 |
| - | 8b | .5376 | .2325 | .2298 | .7675 |
| - | 9b | .5771 | .2253 | .1976 | .7747 |
| - | 10b | .5727 | .2265 | .2008 | .7735 |
| - | 11b | .5743 | .2262 | .1995 | .7738 |
| - | 12b | .5625 | .2302 | .2072 | .7698 |
| | Mean | 0.5578 | 0.2456 | 0.1966 | 0.7583 |
| | Std. dev. | .0207 | .0170 | .0181 | .0114 |
| | | Experimental Animals | | | |
| Strep | 7a | 0.5424 | 0.2123 | 0.2453 | 0.7877 |
| Strep | 8a | .4383 | .2843 | .2775 | .7157 |
| $SO_2$ | 9a | .4972 | .2207 | .2821 | .7793 |
| $SO_2$ | 10a | .5053 | .2344 | .2603 | .7656 |
| $NO_2$ | 11a | .4785 | .2402 | .2814 | .7598 |
| $NO_2$ | 12a | .4767 | .2474 | .2759 | .7526 |

from these 4 sections was divided into 63 squares, about 1 cm. x 1 cm., but representing an area exactly 0.01 cm. x 0.01 cm. in the specimen (as measured by the scale of a stage micrometer photographed at the same magnification). By using a table of random numbers, 10 squares on each photograph were selected for counting. These squares, representing 0.0001 $cm.^2$ in area, contained 10-20 (average 15) alveolar transections (Table 6 and Appendix II). To reduce errors due to visual fatigue, the structures to be counted were marked by pinholes. This prevented skipping a structure or counting it more than once and the pinholes were easily counted and recorded on the back of the photograph.

Since shrinkage does not change the number of alveoli, but only their size calculations were based on "processed" dimensions, without using the shrinkage correction factors.

Estimation of internal surface area. The internal surface area for the lungs of each mouse was estimated by the method of Campbell and Tomkeieff (1952). The overall surface area ($S'_A$) of the double faced alveolar membrane in the processed lung specimen was estimated by the equation

$$S'_A = \frac{4 \cdot V'}{L_m}$$

where $V'$ is the processed volume of the respiratory portion of the lung and $L_m$ is the mean length of the linear intercept. The mean linear intercept ($L_m$) was determined by the formula

$$L_m = \frac{N\ (0.1080\ cm.)}{\Sigma m}$$

where N is the number of fields per specimen, 0.1080 cm. is the length of the sampling line for each field, and $\Sigma m$ is the total number of times the sampling lines intersect the alveolar septa (Chapter II).

TABLE 6

NUMBER OF ALVEOLI IN MOUSE LUNGS

| Treatment | Animal Number | Nm Alveoli per Mouse | n Alveolar Transections: Av. No. Transections per $10^{-4}$ $cm^2$ | n: Std. Dev. | n: Coeff. of Var. | n: Std. Error | N Alveoli per $cm^3$ of Processed Parenchyma | $V_v'$ Volume of Processed Parenchyma |
|---|---|---|---|---|---|---|---|---|
| | | | **Control Animals** | | | | | |
| | | ($\times 10^6$) | # | | | | ($\times 10^6$) | $cm^3$ |
| - | 1a | 11.4 | 15.11 | 1.981 | 13.11% | 0.221 | 43.985 | 0.26 |
| - | 1b | 11.1 | 15.26 | 2.141 | 14.03 | .205 | 44.369 | .25 |
| - | 2a | 9.8 | 15.54 | 2.442 | 15.71 | .272 | 46.690 | .21 |
| - | 2b | 9.5 | 15.29 | 1.840 | 12.03 | .205 | 45.177 | .21 |
| - | 3a | 9.0 | 14.80 | 2.298 | 15.42 | .256 | 42.863 | .21 |
| - | 3b | 10.9 | 14.69 | 2.032 | 13.85 | .226 | 42.053 | .26 |
| - | 4a | 12.0 | 16.01 | 2.530 | 15.80 | .283 | 47.869 | .25 |
| - | 4b | 11.7 | 15.84 | 2.138 | 13.50 | .239 | 46.662 | .25 |
| - | 5a | 11.3 | 15.58 | 2.152 | 13.81 | .241 | 45.324 | .25 |
| - | 5b | 11.2 | 15.46 | 2.470 | 15.97 | .276 | 44.870 | .25 |
| - | 6a | 11.2 | 14.24 | 1.786 | 12.54 | .200 | 40.088 | .28 |
| - | 6b | 10.8 | 13.99 | 1.775 | 12.69 | .199 | 38.492 | .28 |
| - | 7b | 10.2 | 14.35 | 2.571 | 17.92 | .287 | 40.901 | .25 |
| - | 8b | 9.6 | 13.55 | 1.349 | 9.96 | .150 | 36.734 | .26 |
| - | 9b | 9.6 | 15.75 | 1.978 | 12.56 | .221 | 45.830 | .21 |
| - | 10b | 9.1 | 14.69 | 2.434 | 16.57 | .272 | 41.293 | .22 |
| - | 11b | 10.8 | 15.54 | 1.838 | 11.83 | .206 | 44.904 | .24 |
| - | 12b | 11.4 | 14.56 | 2.062 | 14.16 | .231 | 40.861 | .28 |
| | Mean | 10.6 | 15.01 | | | | 43.274 | |
| | | | **Experimental Animals** | | | | | |
| | | ($\times 10^6$) | # | | | | ($\times 10^6$) | $cm^3$ |
| Strep | 7a | 11.6 | 16.21 | 2.273 | 14.02% | 0.254 | 50.231 | 0.23 |
| Strep | 8a | 6.9 | 14.74 | 2.381 | 16.15 | .266 | 43.146 | .16 |
| $SO_2$ | 9a | 8.7 | 14.33 | 1.935 | 13.50 | .216 | 39.635 | .22 |
| $SO_2$ | 10a | 9.6 | 14.79 | 2.207 | 14.92 | .246 | 41.869 | .23 |
| $NO_2$ | 11a | 10.1 | 14.41 | 1.984 | 13.77 | .222 | 40.496 | .25 |
| $NO_2$ | 12a | 11.2 | 13.93 | 2.019 | 14.49 | .226 | 38.628 | .29 |

Each of the random sections from each specimen was examined under high magnification (6x eyepiece, 45x objective). A reticle ruled in 1 mm. squares was placed in the focal plane (on the field diaphragm) of the 6x Huygenian eyepiece. Two vertical and two horizontal lines in this reticle, representing a total length of 0.1080 cm. (1080 $\mu$) in the section (as measured by a stage micrometer), were used as the sampling lines. Sampling lines at right angles to each other were used to compensate for possible "compression" artifacts due to sectioning (Weibel, 1963b, p. 38). In each field the number of intersections of these lines with alveolar septa was usually about 34-40 (Table 7 and Appendix III).

Five fields in each section were selected by random placements of the mechanical stage.

The surface areas ($S'_A$) of the processed specimens were converted to surface areas ($S_A$) at the "standard" (1.0 $cm.^3$) volume by use of the correction factors for areal dimensions listed in Table 1. In other words, $S'_A$ is the surface area actually determined from measurements on the processed specimen. $S_A$ is an estimate of what the surface area would have been if the same specimen could have been measured, before shrinkage, at the "standard" volume of 1.0 $cm.^3$

## Statistical Analysis of Differences Between Paired Animals

The data included 12 morphometric values for each of the 24 animals in this study. Within each pair, the values for animal "b" were substracted from the values for animal "a", giving a sample composed of differences between paired animals for each of the 12 morphometric values.

TABLE 7

INTERNAL SURFACE AREA OF MOUSE LUNGS

| Treatment | Animal Number | SA Internal Surface Area at Std. Vol. of 1.0 $cm^3$ | I Alveolar Septal Intercepts: Avg. No. Intercepts per 0.108 cm | Std. Dev. | Coeff. of Var. | Std. Error | $L_m$ Mean Length Between Intercepts | $SA'$ Internal Surface Area of Processed Specimen |
|---|---|---|---|---|---|---|---|---|
| **Control Animals** | | | | | | | | |
| | | $cm^2$ | # | | | | $10^{-4}$ cm | $cm^2$ |
| - | 1a | 780 | 36.38 | 2.402 | 6.60% | 0.298 | 29.69 | 350.3 |
| - | 1b | 740 | 36.23 | 2.572 | 7.10 | .319 | 29.81 | 335.5 |
| - | 2a | 720 | 36.56 | 1.512 | 4.14 | .200 | 29.54 | 284.4 |
| - | 2b | 710 | 36.23 | 2.509 | 6.93 | .347 | 29.81 | 281.8 |
| - | 3a | 750 | 38.10 | 3.384 | 8.88 | .404 | 28.35 | 296.3 |
| - | 3b | 770 | 35.97 | 3.529 | 10.10 | .456 | 30.03 | 346.3 |
| - | 4a | 780 | 38.12 | 2.272 | 5.96 | .374 | 28.33 | 353.0 |
| - | 4b | 760 | 36.94 | 2.519 | 6.82 | .415 | 29.24 | 342.0 |
| - | 5a | 770 | 37.26 | 1.840 | 4.94 | .303 | 28.99 | 344.9 |
| - | 5b | 780 | 37.72 | 1.801 | 4.77 | .297 | 28.63 | 349.3 |
| - | 6a | 750 | 35.40 | 2.321 | 6.56 | .382 | 30.51 | 367.1 |
| - | 6b | 780 | 35.78 | 1.846 | 5.16 | .304 | 30.18 | 371.1 |
| - | 7b | 710 | 34.62 | 2.702 | 7.08 | .382 | 31.20 | 320.5 |
| - | 8b | 710 | 33.12 | 2.847 | 8.60 | .403 | 32.61 | 318.9 |
| - | 9b | 730 | 37.98 | 2.025 | 5.33 | .334 | 28.44 | 295.4 |
| - | 10b | 750 | 37.44 | 2.109 | 6.71 | .347 | 28.85 | 305.0 |
| - | 11b | 780 | 37.46 | 1.841 | 4.92 | .303 | 28.83 | 330.0 |
| - | 12b | 820 | 37.86 | 2.010 | 5.31 | .331 | 28.53 | 392.6 |
| | Mean | 755 | 36.62 | | | | 29.53 | 332.6 |
| **Experimental Animals** | | | | | | | | |
| | | $cm^2$ | # | | | | $10^{-4}$ cm | $cm^2$ |
| Strep | 7a | 660 | 34.95 | 3.002 | 8.59% | 0.344 | 30.90 | 297.7 |
| Strep | 8a | 410 | 31.31 | 2.889 | 9.23 | .303 | 34.49 | 185.6 |
| $SO_2$ | 9a | 710 | 36.70 | 2.181 | 5.94 | .359 | 29.43 | 299.0 |
| $SO_2$ | 10a | 710 | 35.72 | 1.333 | 3.73 | .220 | 30.24 | 304.8 |
| $NO_2$ | 11a | 730 | 36.32 | 2.015 | 5.65 | .332 | 29.74 | 336.2 |
| $NO_2$ | 12a | 770 | 34.96 | 1.737 | 4.97 | .286 | 30.89 | 375.5 |

It was assumed that the differences between paired animals in the Control Pairs would be normally distributed about a true mean of zero (Snedecor, 1956, p. 77). For each of the 12 morphometric values, the means ($\bar{d}$) of the differences ($d_i$) for the 6 Control Pairs were calculated (Tables 8, 9, 10). Standard deviations ($S_d$) and standard errors of the means ($S_{\bar{d}}$) were calculated. For each of these 12 values the hypothesis that the true mean ($\mu$) equals zero was subjected to a t-test as follows:

$$H_o: \mu = 0$$

$$t = \frac{\bar{d} - \mu}{\frac{S_d}{\sqrt{n}}} = \frac{\bar{d}}{S_{\bar{d}}} .$$

The critical value of t at the 0.05 level, with 5 degrees of freedom is 2.571. If the t ratio calculated above did not exceed $\pm$ 2.571, we "failed to reject" the null hypothesis that the true mean of the differences was zero. As indicated in Tables 8, 9 and 10, this was the case for all 12 morphometric values.

If the differences were normally distributed about a mean of zero, then differences greater than

$$0 \pm t\ (.05,\ 5\text{d.f.})\ S_d$$

would be uncommon (expected to occur by chance alone only 5 times in 100).

Differences between paired animals for the Experimental Pairs are listed in Tables 8, 9 and 10. If the experimental treatments had produced no effect, these differences should be samples from the same populations as the differences in the Control Pairs. Asterisks mark the differences than are greater than $0 \pm (2.571)\ S_d$. The probability is only 5 in 100 that differences as large as these would happen through chance alone.

TABLE 8

DIFFERENCES BETWEEN PAIRED ANIMALS: LUNG COMPONENTS AS A FRACTION OF WHOLE LUNG

| Treatment of Animal | | Pair No. | Differences Between Paired Animals | | | | | |
|---|---|---|---|---|---|---|---|---|
| a | b | | $\frac{A}{T}$ Alveolar Air | $\frac{D}{T}$ Alveolar Duct Air | $\frac{S}{T}$ Alveolar Septa | $\frac{C}{T}$ Bronchi, Blood Vessels, etc. | $\frac{I}{T}$ Inflam. and Lymphoid Tissue | $\frac{A+D+S}{T}$ Parenchyma |
| | | | **Control Pairs** | | | | | |
| - | - | 1 | +0.0012 | +0.0131 | +0.0087 | -0.0325 | +0.0094 | +0.0232 |
| - | - | 2 | -0.0060 | +0.0140 | +0.0037 | -0.0116 | 0.0000 | +0.0116 |
| - | - | 3 | -0.0284 | -0.0144 | -0.0029 | +0.0155 | +0.0155 | -0.0456 |
| - | - | 4 | -0.0397 | +0.0014 | +0.0258 | +0.0055 | -0.0030 | -0.0025 |
| - | - | 5 | +0.0021 | -0.0007 | -0.0009 | 0.0000 | -0.0005 | +0.0005 |
| - | - | 6 | -0.0256 | +0.0122 | -0.0066 | +0.0219 | -0.0019 | -0.0200 |
| Total | | | -0.0964 | +0.0256 | +0.0278 | -0.0012 | +0.0341 | -0.0328 |
| Mean | | | -0.0161 | +0.0043 | +0.0046 | -0.0002 | +0.0057 | -0.0055 |
| Std. dev. | | | ±0.0175 | ±0.0111 | ±0.0117 | ±0.0197 | ±0.0128 | ±0.0244 |
| Std.error | | | ±0.0071 | ±0.0045 | ±0.0048 | ±0.0080 | ±0.0052 | ±0.0100 |
| $H_o:\mu=0$ $t=\frac{\bar{d}}{S_{\bar{d}}}$ | | | -2.268 | +0.956 | +0.958 | -0.025 | +1.096 | -0.550 |
| $t_{.05}S_d$ | | | ±0.0405 | ±0.0285 | ±0.0301 | ±0.0506 | ±0.0329 | ±0.0627 |
| | | | **Experimental Pairs** | | | | | |
| Strep | - | 7 | -0.0123 | -0.0608* | -0.0046 | +0.0378 | +0.0399* | -0.0778* |
| Strep | - | 8 | -0.2224* | -0.0445* | -0.0458* | +0.0166 | +0.2962* | -0.3127* |
| $SO_2$ | - | 9 | -0.0499* | -0.0025 | +0.0755* | -0.0281 | 0.0000 | +0.0281 |
| $SO_2$ | - | 10 | -0.0729* | -0.0010 | +0.0416* | +0.0325 | 0.0000 | -0.0325 |
| $NO_2$ | - | 11 | -0.0945* | +0.0052 | +0.0616* | +0.0205 | +0.0074 | -0.0279 |
| $NO_2$ | - | 12 | -0.0607* | +0.0201 | +0.0637* | -0.0275 | +0.0044 | -0.0231 |

*$P < 0.05$

TABLE 9

DIFFERENCES BETWEEN PAIRED ANIMALS: LUNG COMPONENTS AS A FRACTION OF LUNG PARENCHYMA

| Treatment of Animal | | Pair Number | Differences Between Paired Animals | | | |
|---|---|---|---|---|---|---|
| a | b | | $\frac{A}{A+D+S}$ Alveolar Air | $\frac{D}{A+D+S}$ Alveolar Duct Air | $\frac{S}{A+D+S}$ Alveolar Septa | $\frac{A+S}{A+D+S}$ Alveoli (Air + Septa |
| **Control Pairs** | | | | | | |
| - | - | 1 | -0.0137 | +0.0087 | +0.0050 | -0.0087 |
| - | - | 2 | -0.0146 | +0.0128 | -0.0118 | -0.0128 |
| - | - | 3 | -0.0024 | -0.0035 | +0.0059 | +0.0035 |
| - | - | 4 | -0.0464 | +0.0146 | +0.0318 | -0.0146 |
| - | - | 5 | +0.0022 | -0.0010 | -0.0012 | +0.0010 |
| - | - | 6 | -0.0176 | +0.0205 | -0.0029 | -0.0205 |
| | | Total | -0.0925 | +0.0521 | -0.0268 | -0.0521 |
| | | Mean | -0.0154 | +0.0087 | +0.0045 | -0.0087 |
| | | Std.dev. | $\pm$0.0170 | $\pm$0.0093 | $\pm$0.0155 | $\pm$0.0093 |
| | | Std. error | $\pm$0.0069 | $\pm$0.0038 | $\pm$0.0063 | $\pm$0.0038 |
| | | $H_o:\mu=0$ | | | | |
| | | $t = \frac{\bar{d}}{S_{\bar{d}}}$ | -.2.232 | +2.289 | +0.714 | -2.289 |
| | | $t_{.05}S_d$ | $\pm$0.0437 | $\pm$0.0239 | $\pm$0.0399 | $\pm$0.0239 |
| **Experimental Pairs** | | | | | | |
| Strep | - | 7 | +0.0048 | -0.0202 | +0.0255 | +0.0202 |
| Strep | - | 8 | -0.0686* | +0.0194 | +0.0483* | +0.9194 |
| $SO_2$ | - | 9 | -0.0799* | -0.0046 | +0.0845* | +0.0046 |
| $SO_2$ | - | 10 | -0.0674* | +0.0079 | +0.0595* | -0.0079 |
| $NO_2$ | - | 11 | -0.0958* | +0.0140 | +0.0819* | -0.0140 |
| $NO_2$ | - | 12 | -0.0858* | +0.0172 | +0.0687* | -0.0172 |

*$P < 0.05$

TABLE 10

DIFFERENCES BETWEEN PAIRED ANIMALS: INTERNAL SURFACE AREA AND NUMBER OF ALVEOLI

| Treatment of Animal | | Pair Number | Differences Between Paired Animals | |
|---|---|---|---|---|
| a | b | | Nm Alveoli per Mouse ($\times 10^6$) | SA Internal Surface Area ($cm^2$) |
| | | | Control Pairs | |
| - | - | 1 | +0.3 | +40 |
| - | - | 2 | +0.3 | +10 |
| - | - | 3 | -1.9 | -20 |
| - | - | 4 | +0.3 | +20 |
| - | - | 5 | +0.1 | -10 |
| - | - | 6 | +0.4 | -30 |
| | | Total | -0.5 | +10 |
| | | Mean | -0.08 | + 1.7 |
| | | Std. dev. | $\pm$0.90 | $\pm$26.4 |
| | | Std.error | $\pm$0.37 | $\pm$10.8 |
| | | $H_o: \mu = 0$ | | |
| | | $t = \frac{\bar{d}}{s_{\bar{d}}}$ | -2.162 | + 0.157 |
| | | $t_{.05} s_d$ | $\pm$2.3 | $\pm$68 |
| | | | Experimental Pairs | |
| Strep | - | 7 | +1.4 | -50 |
| Strep | - | 8 | -2.7* | -300* |
| $SO_2$ | - | 9 | -0.9 | -40 |
| $SO_2$ | - | 10 | +0.6 | -20 |
| $NO_2$ | - | 11 | -0.7 | -50 |
| $NO_2$ | - | 12 | -0.2 | -50 |

*$P < 0.05$

# CHAPTER IV

# RESULTS

## Morphometry of Mouse Lungs

Numerical expressions of some aspects of the pulmonary anatomy and pathology of 24 white mice have been presented in Tables 4, 5, 6, and 7.

### Volumetric Proportions

Table 4 lists 5 separate categories of lung components expressed as decimal fractions of whole lung volume for each of the 24 mice. Converting these decimal fractions to percentages, the lungs of a mouse (control animal number 4b, for example) can be characterized as consisting of: alveolar air, 48.67%; alveolar duct air, 19.80%; alveolar septa, 14.23%; bronchi, blood vessels, connective tissues, 17.00%; and lymphoid aggregates, 0.30%. The sum of these 5 categories is 100%. The sum of the first 3 categories, designated "parenchyma" or "respiratory portion" of the lung, is listed in the last column of Table 4. The processed lung volume, multiplied by this fraction, gives the volume of processed lung parenchyma that is listed in the last column of Table 6.

The amount of pneumonia present in the animals given streptococci is expressed in quantitative terms in Table 4. When sacrificed 48 hours

after being infected, animal 7a had an inflammatory exudate involving about 4% of its lung volume. Animal number 8a, sacrificed 72 hours after being infected, had involvement of almost 30% of its lung.

The alterations in the lungs of the other experimental animals were less striking, but they appear to differ from the controls in regards to the alveolar air and alveolar septal fractions. Statistical tests for significant differences will be discussed in a later section, but it can be seen in Table 4 that 6 of 6 fractions for alveolar air lie below the lowest control value and 4 of 6 fractions for alveolar septa lie above the highest control value.

Table 5 expresses 3 categories of lung components as fractions of the lung parenchyma. The lung parenchyma of animal 4b, for example, can be characterized as follows: alveolar air, 58.85%; alveolar duct air, 23.94%; and alveolar septa, 17.21%. The sum of these 3 categories is 100%. The sum of the first and third categories, expressing alveoli (lumens and walls) as a fraction of lung parenchyma, is listed in the last column of Table 5.

The experimental animals appear to differ from controls in Table 5 in about the same way as in Table 4. Five out of 6 of them have fractions for alveolar air below the lowest control value and 6 out of 6 have fractions for alveolar septa that are higher than the highest control value.

## Number of Alveoli

Estimates of the number of alveoli for each of the 24 mice are listed in Table 6. The mean value for the 18 control animals is 10.6 million alveoli. The rather broad range of values, 9.1 million

to 12 million, may represent variability among the animals, but is probably the result of errors in measurement, since a difference of only 0.05 $cm.^3$ in the determination of the processed lung volume would result in a difference of about 2 million in the estimated number of alveoli.

The estimates for two experimental animals lie below the range observed in the controls. The value listed for animal 8a, which had received streptococci 72 hours prior to sacrifice, includes only the aerated alveoli. Alveoli filled with inflammatory exudate are not included in the count.

### Internal Surface Area

Estimates of the internal surface area, expressed as the area that would exist if the specimen were inflated to the standard volume of 1.0 $cm.^3$, are listed in Table 7. The mean value for the 18 control animals is 755 $cm.^2$ with a range from 710 to 810 $cm.^2$

Only the 2 experimental animals with streptococcal pneumonia lie below the range observed for the control animals.

### Differences Between Paired Animals in Control and Experimental Pairs

Differences between paired animals in the control pairs (control minus control) and experimental pairs (experimental minus control) have been presented in Tables 8, 9 and 10. The statistical analyses tested the hypotheses that the differences in each experimental pair are samples from the same populations as the differences in the control pairs. Asterisks mark the ones that are <u>not</u> the same, (that is, the chances are less than 5 in 100 that they <u>are</u> the same). This, then, is objective

evidence that the experimental treatments produced alterations in the experimental animals' lungs.

## Volumetric Proportions

Table 8 shows asterisks for 16 of the 36 items listed for the experimental pairs. As expected, both of the experimental pairs in which the experimental member received streptococci (7a, 8a) had significant differences (increases) in the amounts of inflammatory and lymphoid tissue. The other experimental pairs showed significant differences in the alveolar air and alveolar septal fractions. The signs (+ or -) indicate that, in each of the last 4 pairs, the alveolar air fraction of the experimental animal was less than its control, and the alveolar septal fraction of the experimental animal was greater than its control.

Table 9 shows the same changes as Table 8 for the 4 pairs in which the experimental animal received $SO_2$ or $NO_2$.

## Number of Alveoli

The only pair with a significant difference (decrease) in number of alveoli (Table 10) is the one in which the treated member had severe streptococcal pneumonia (pair 8). Since only aerated alveoli were counted, this difference represents alveoli filled with inflammatory exudate.

## Internal Surface Area

The only pair with a significant difference (decrease) in internal surface area is also pair number 8 (severe streptococcal pneumonia). It should be noted, however, that in every one of the other experimental

pairs, the value for the treated animal is less than the value for its control.

## Histopathology

### Control Animals

The only "abnormality" encountered more than once in histopathologic examinations of the lungs of the control mice was the presence of scattered, peri-vascular and peri-bronchial lymphoid aggregates. These small aggregates, present in 6 of 18 control animals, probably should be considered normal lung components. One control animal (number 3a) had a focal, acute pneumonia, characterized by intra-alveolar and intra-bronchial exudates of fibrin and polymorphonuclear leucocytes.

### Experimental Animals

Both experimental animals that received streptococci (7a, 8a) developed acute exudative pneumonia. The process was much more extensive in the animal sacrificed at 72 hours than in the one sacrificed 48 hours after exposure.

The lungs of the 2 experimental animals exposed to sulfur dioxide (9a, 10a) could not be distinguished, with certainty, from the lungs of the controls. The small blood vessels and alveolar septal capillaries were engorged, but this change was also seen in many of the controls.

The lungs of both experimental animals exposed to oxides of nitrogen (11a, 12a) developed focal, cellular thickenings of alveolar septa, mainly in alveoli adjacent to terminal bronchi and alveolar

ducts. Many of these same alveoli were more than half filled with desquamated cells or alveolar phagocytes.

# CHAPTER V

## DISCUSSION

This study used morphometric methods to objectively evaluate the effects of noxious agents on the lungs of laboratory animals. In some instances (experimental animals 9a and 10a) it was possible to detect alterations that would have been overlooked or considered equivocal by routine histopathologic evaluations. In other instances (experimental animals 7a and 8a) the alterations were obvious, even in the gross specimens, but it was possible to quantitate the changes by using these techniques. However, the switch from qualitative to quantitative terms in morphologic descriptions required a tremendous amount of additional time and effort. The value of the information gained from the detailed quantitative study of a few specimens must be weighed against the limited number of specimens that cen be studied in a given period of time.

In addition to the detection and quantitation of experimentally induced alterations, there are several other uses for morphometric data in studies of experimental pulmonary pathology.

It is important to consider quantitative aspects of the normal lung morphology of various laboratory animals when selecting a species for a particular experiment. For instance, the size, geometry, and anatomic arrangement of airways could be very important factors

influencing the retention and localization of particulate matter (Yevich, 1965). Soluble or reactive gases, that penetrate to the alveolar level and produce lesions there in small animals, might be removed from the air-stream in the upper airways of larger animals.

The need for caution in extrapolating from animal experiments to humans would be emphasized if comparisons of their quantitative lung morphology were available. Table 11 compares normal human lungs and normal mouse lungs.

TABLE 11

COMPARISON OF LUNGS OF MAN AND MOUSE

| | Man[a] | Mouse |
|---|---|---|
| Number of Alveoli | 300,000,000 | 10,600,000 |
| Generations of Airway Branchings | 22-23 | 7-9[b] |
| Internal Surface Area | 75-80 $m^2$ | 0.07-0.08 $m^2$ |
| Diameter of Alveoli | 200-300 $\mu$ | 30-50 $\mu$ |
| Lung Volume | 5000 $cm^3$ | 1 $cm^3$ |

[a]Weibel, 1963b [b]Krahl, 1964

Morphometric techniques, such as point counting volumetry, can be employed to reduce subjective bias in some histopathologic evaluations even if the results are to be recorded or reported in descriptive and qualitative terms. A few minutes spent tallying a few representative fields can help the microscopist decide on some reasonably objective and reproducible standards for terms such as "mild", "moderate", and "severe", or "slight" and "extensive".

In a similar way, point counting can be used to reduce arbitrariness in quasi-quantitative techniques where lesions are graded as "+", "++","+++", etc. If the grading system is based on the amount (volume) of a substance or type of tissue change, occasional spot checks by the point counting method can determine if the same criteria for the various grades are being maintained throughout the series.

# CHAPTER VI

## SUMMARY

The objective of this study was to explore the possibilities of using morphometric (morphologic measurement) techniques to supplement conventional descriptive histopathologic examinations in the assay of experimental lung alterations in mice. It was undertaken because of a need for objective methods of detecting and evaluating subtle changes in the lungs of experimental animals exposed to atmospheres that contain low levels of noxious agents.

The techniques were based on principles pertaining to two-dimensional samples of three-dimensional structures, originally developed by geologists, but recently used for quantitative studies of human lungs and other organs. From measurements made microscopically on histologic sections of mouse lungs, estimates of (1) relative volumes of anatomic components, (2) number of alveoli, and (3) internal surface area were obtained.

Twelve pairs of mice, matched according to age, sex, and weight were studied. Six pairs were Control Pairs in which neither member was exposed to a noxious agent. Six pairs were Experimental Pairs in which one member, chosen by lot, was an experimental animal (exposed to a noxious agent) and the other member was its paired control. Statistical analyses were based on differences between paired animals.

Streptococcal pneumonia was produced in two experimental animals by intra-nasal insufflation of droplets of a broth culture of the bacteria. Two experimental animals were exposed to sulfur dioxide (100 ppm) and two were exposed to oxides of nitrogen (15-20 ppm) for eight days in controlled environment chambers.

By point counting volumetry, statistically significant differences in the alveolar air fractions (decreased) and alveolar septal fractions (increased) could be demonstrated in the lungs of the mice exposed either to sulfur dioxide or oxides of nitrogen. On routine histopathologic examinations of the same specimens, localized proliferative changes were observed in the lungs of animals exposed to oxides of nitrogen, but the lungs of animals exposed to sulfur dioxide could not be distinguished from the controls.

The alterations in the lungs of the mice exposed to streptococci were easily detected, even by gross inspection, but the morphometric techniques permitted determinations of the percentage of lung volume occupied by the pneumonia, and of number and surface area of uninvolved alveoli.

Changes reflected by point counting volumetry were more sensitive indicators of experimentally induced lung alterations than were changes in number of alveoli or internal surface area.

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## APPENDIX I

### DIFFERENTIAL POINT COUNTS OF INDIVIDUAL MICROSCOPIC FIELDS FOR LUNGS OF ONE MOUSE (ANIMAL NUMBER 4b)

| A Alveolar Air | D Alveolar Duct Air | S Alveolar Septa | C Bronchi, Blood Vessels, etc. | I Inflam. and Lymphoid Tissue | T Total Points Counted |
|---|---|---|---|---|---|
| | | Slide # 188 | | | |
| 12 | 8 | 3 | 10 | 0 | 33 |
| 13 | 3 | 5 | 4 | 0 | 25 |
| 12 | 7 | 3 | 0 | 0 | 22 |
| 16 | 4 | 2 | 3 | 0 | 25 |
| 19 | 7 | 4 | 0 | 0 | 30 |
| 14 | 5 | 3 | 3 | 0 | 25 |
| 14 | 6 | 2 | 1 | 0 | 23 |
| 14 | 4 | 3 | 5 | 0 | 26 |
| 11 | 6 | 2 | 1 | 0 | 20 |
| 13 | 4 | 3 | 4 | 0 | 24 |
| 8 | 4 | 2 | 6 | 0 | 20 |
| | | Slide # 295 | | | |
| 11 | 7 | 3 | 2 | 0 | 23 |
| 13 | 6 | 4 | 0 | 0 | 23 |
| 12 | 5 | 3 | 5 | 0 | 25 |
| 14 | 3 | 2 | 1 | 0 | 20 |
| 12 | 3 | 1 | 8 | 1 | 25 |
| 11 | 3 | 4 | 2 | 0 | 20 |
| 13 | 4 | 4 | 1 | 0 | 22 |
| 15 | 6 | 2 | 2 | 0 | 25 |
| 8 | 3 | 2 | 7 | 0 | 20 |
| 7 | 2 | 3 | 9 | 0 | 21 |
| 8 | 4 | 3 | 3 | 2 | 20 |
| 8 | 5 | 5 | 2 | 0 | 20 |
| 10 | 6 | 3 | 5 | 0 | 24 |
| 18 | 7 | 6 | 6 | 0 | 37 |
| 12 | 10 | 5 | 8 | 0 | 35 |

APPENDIX I - Continued

| A | D | S | C | I | T |
|---|---|---|---|---|---|
| | | Slide # 436 | | | |
| 20 | 8 | 4 | 2 | 0 | 34 |
| 9 | 4 | 4 | 3 | 0 | 20 |
| 9 | 3 | 2 | 8 | 0 | 22 |
| 20 | 8 | 4 | 3 | 0 | 35 |
| 13 | 3 | 4 | 2 | 0 | 22 |
| 10 | 4 | 3 | 8 | 0 | 25 |
| 7 | 3 | 3 | 10 | 0 | 23 |
| 11 | 4 | 3 | 2 | 0 | 20 |
| 9 | 7 | 3 | 1 | 0 | 20 |
| 19 | 5 | 2 | 4 | 0 | 30 |
| 12 | 6 | 1 | 6 | 0 | 25 |
| 13 | 4 | 7 | 2 | 0 | 26 |
| 8 | 4 | 3 | 6 | 0 | 21 |
| 16 | 3 | 4 | 6 | 0 | 29 |
| 15 | 3 | 4 | 1 | 0 | 23 |
| 12 | 4 | 5 | 4 | 0 | 25 |
| | | Slide # 520 | | | |
| 15 | 5 | 7 | 3 | 0 | 30 |
| 10 | 3 | 4 | 12 | 0 | 29 |
| 6 | 10 | 4 | 5 | 0 | 25 |
| 13 | 5 | 3 | 3 | 0 | 25 |
| 10 | 5 | 3 | 3 | 0 | 21 |
| 10 | 5 | 2 | 5 | 0 | 22 |
| 8 | 1 | 4 | 7 | 0 | 20 |
| 10 | 4 | 6 | 0 | 0 | 20 |
| 13 | 3 | 2 | 5 | 0 | 23 |
| 15 | 2 | 4 | 4 | 0 | 25 |
| 12 | 2 | 2 | 9 | 0 | 25 |
| 13 | 4 | 3 | 4 | 0 | 24 |
| 12 | 8 | 4 | 1 | 0 | 25 |
| 11 | 6 | 3 | 2 | 0 | 22 |
| 11 | 3 | 3 | 4 | 0 | 21 |

APPENDIX I - Continued

| A | D | S | C | I | T |
|---|---|---|---|---|---|
| | | Slide # 540 | | | |
| 10 | 7 | 3 | 1 | 0 | 21 |
| 7 | 2 | 2 | 13 | 0 | 24 |
| 21 | 8 | 2 | 4 | 0 | 35 |
| 11 | 3 | 1 | 6 | 0 | 21 |
| 19 | 9 | 8 | 2 | 0 | 38 |
| 13 | 5 | 1 | 3 | 0 | 22 |
| 11 | 2 | 2 | 10 | 0 | 25 |
| 12 | 11 | 5 | 3 | 0 | 31 |
| 7 | 8 | 1 | 6 | 0 | 22 |
| 10 | 7 | 4 | 3 | 0 | 24 |
| 10 | 3 | 1 | 11 | 0 | 25 |
| 14 | 3 | 2 | 6 | 0 | 25 |
| 15 | 8 | 3 | 1 | 0 | 27 |
| | | Slide # 594 | | | |
| 10 | 5 | 3 | 3 | 0 | 21 |
| 8 | 2 | 3 | 7 | 0 | 20 |
| 13 | 6 | 8 | 5 | 0 | 32 |
| 10 | 2 | 2 | 8 | 0 | 22 |
| 8 | 3 | 5 | 4 | 0 | 20 |
| 10 | 9 | 3 | 3 | 0 | 25 |
| 13 | 4 | 3 | 1 | 0 | 21 |
| 13 | 8 | 9 | 4 | 0 | 34 |
| 11 | 5 | 5 | 4 | 0 | 25 |
| | | Slide # 638 | | | |
| 10 | 9 | 5 | 1 | 0 | 25 |
| 13 | 4 | 7 | 7 | 0 | 31 |
| 4 | 5 | 2 | 12 | 1 | 24 |
| 14 | 4 | 2 | 9 | 0 | 29 |
| 15 | 4 | 8 | 5 | 0 | 32 |
| 13 | 0 | 5 | 3 | 0 | 21 |
| 9 | 6 | 2 | 7 | 1 | 25 |
| 11 | 2 | 4 | 8 | 0 | 25 |
| 11 | 5 | 6 | 3 | 0 | 25 |

APPENDIX I - Continued

| A | D | S | C | I | T |
|---|---|---|---|---|---|
| | | Slide # 791 | | | |
| 10 | 2 | 5 | 4 | 1 | 22 |
| 17 | 2 | 3 | 2 | 0 | 24 |
| 15 | 10 | 2 | 4 | 0 | 31 |
| 13 | 8 | 4 | 2 | 0 | 27 |
| 19 | 7 | 4 | 2 | 0 | 35 |
| 11 | 2 | 5 | 4 | 0 | 22 |
| | | Slide # 864 | | | |
| 14 | 3 | 4 | 1 | 0 | 22 |
| 13 | 4 | 2 | 1 | 0 | 20 |
| 13 | 7 | 2 | 1 | 0 | 23 |
| 11 | 3 | 2 | 7 | 0 | 23 |
| 11 | 6 | 5 | 1 | 0 | 23 |
| 10 | 7 | 4 | 1 | 1 | 23 |
| 18 | 8 | 6 | 2 | 0 | 34 |
| 19 | 6 | 5 | 6 | 0 | 36 |
| | | Slide # 1062 | | | |
| 13 | 6 | 2 | 0 | 0 | 21 |
| 11 | 7 | 5 | 0 | 0 | 23 |
| 12 | 7 | 3 | 1 | 0 | 23 |
| 8 | 2 | 1 | 11 | 0 | 22 |
| 19 | 3 | 6 | 8 | 0 | 36 |
| | | Grand Totals | | | |
| 1303 | 530 | 381 | 455 | 8 | 2677 |

## APPENDIX II

### ALVEOLAR TRANSECTIONS PER 0.0001 $cm^2$ FOR LUNGS OF ONE MOUSE (ANIMAL NUMBER 4b)

| Slide No. 436 | Slide No. 520 | Slide No. 540 | Slide No. 638 |
|---|---|---|---|
| 14 | 11 | 13 | 13 |
| 14 | 11 | 15 | 13 |
| 14 | 12 | 15 | 15 |
| 15 | 13 | 16 | 15 |
| 15 | 15 | 16 | 17 |
| 16 | 15 | 17 | 17 |
| 16 | 16 | 17 | 17 |
| 16 | 16 | 18 | 17 |
| 17 | 18 | 18 | 17 |
| 19 | 19 | 20 | 20 |
| 13 | 15 | 13 | 13 |
| 14 | 15 | 13 | 14 |
| 14 | 16 | 14 | 14 |
| 14 | 17 | 15 | 14 |
| 14 | 17 | 15 | 15 |
| 16 | 17 | 16 | 15 |
| 17 | 17 | 17 | 15 |
| 18 | 17 | 18 | 16 |
| 19 | 18 | 19 | 17 |
| 20 | 20 | 21 | 17 |

Grand total 1267
Mean 15.84

## APPENDIX III

### ALVEOLAR SEPTAL INTERCEPTS FOR LUNGS OF ONE MOUSE (ANIMAL NUMBER 4b)

| Slide No. 188 | Slide No. 295 | Slide No. 436 | Slide No. 520 | Slide No. 540 | Slide No. 594 | Slide No. 638 | Slide No. 791 | Slide No. 864 | Slide No. 1062 |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 35 | 35 | 33 | 40 | 38 | 37 | 34 | 36 | 35 |
| 40 | 33 | 36 | 37 | 36 | 40 | 35 | 44 | 40 | 37 |
| 38 | 36 | 37 | 38 | 38 | 41 | 34 | 40 | 38 | 36 |
| 36 | 40 | 36 | 38 | 34 | 34 | 38 | 36 | 37 | 37 |
| 39 | 38 | 41 | 33 | 38 | 37 | 36 | 36 | 39 | 37 |
| | | Grand total | 1847 | | | | | | |
| | | Mean | 36.94 | | | | | | |