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Evaluation of a simple method of sampling the lung for quantitative histological analysis

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In a previous paper (Dunnill, 1962), various quantitative methods were described for use in studying the pathological anatomy of the lung. These methods were developed from those used in the normal lung by E. R. Weibel, and their theoretical basis has been described fully by him (Weibel, 1963). An essential preliminary procedure was the selection of blocks of lung tissue to be sectioned for histological analysis, and a method of stratified random sampling was described. It is the purpose of this paper to assess the efficiency of this method in a series of samples from a normal and an emphysematous subject.

MATERIAL AND METHODS

Normal lungs were obtained from a woman, 55 years old, who died of a head injury in an automobile accident. There was no recognizable lung disease present. The abnormal lungs came from a woman of 60 years who suffered from a severe degree of centrilobular emphysema. The centrilobular spaces were scattered fairly uniformly throughout both lungs and accounted for 43% of the lung volume, as determined by the point-counting method on the gross specimen (Dunnill, 1962). The lungs from both cases were treated in an identical manner. They were removed from the cadaver with extreme care to avoid puncturing the pleura and were then inflated with air to what was estimated as total lung capacity. The volumes of the inflated lungs were measured by water displacement, the mean of five measurements being taken. They were fixed with formalin steam by the method of Weibel and Vidone (1961) and allowed to float in Zenker's solution overnight.

The volumes of the fixed organs were then measured by water displacement. The determination of the fresh and the fixed volumes allowed for the calculation of a factor, f^3, for the correction of fixed volumes to fresh volumes, the values f^2 and f being the correction factors for area and linear measurements, respectively, on the assumption that shrinkage is equal in each dimension. In both these cases the value of f^3 was 1·82. The lungs were then cut through the hilum into a series of 1 cm. thick parallel slices. The volume of the parenchyma, as opposed to the non-parenchyma consisting of blood vessels and airways greater than 0·1 cm. in diameter, was determined by the simple point-counting method described by Dunnill (1962). The slices were placed side by side on a flat surface and each slice was covered completely with a piece of cellophane on which was drawn a grid composed of squares of side 1 cm. The corners of each square were perforated so that the resulting hole would admit a pin head. The squares were numbered consecutively. If 20 blocks were required and 10 slices were present, the first square was selected by means of the random number table and the second square by addition of a constant number to the chosen random number.

This procedure avoids the unlikely, but possible, event of sampling two adjacent areas which might occur with pure random sampling employing no system of stratification. Furthermore, blocks from the hilum must be excluded because large bronchi and blood vessels are present and there is little or no parenchyma. When a square had been selected a pin was passed through the perforation at the top left-hand corner of the square, and, when all the squares had been chosen, the grids were removed, leaving the pins in situ. Blocks of tissue were cut out of the lung with the pin placed arbitrarily at the top left-hand corner of the block. The size of the blocks was kept as uniform as possible, 2·8 × 2·0 × 1·0 cm. being found to be convenient. Each block was measured, processed, and embedded in paraffin, and sections were cut at 5 μ thickness. The sections were stained by the Masson trichrome or P.A.S. methods. One section from each block was used for the subsequent histo-

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logical measurements. The area occupied by the sections on the slide was measured, and this, when compared with the area of the cut surface of the fixed block, gave a factor for converting areas of processed tissue to fixed tissue. From this factor, p^2, similar factors, p^3 for volume and p for linear dimensions, were calculated.

HISTOLOGICAL ANALYSIS

Volume proportions The volume proportions of the alveolar air, respiratory duct air, tissue, and vessels were determined, using the point-counting integrating eyepiece of Zeiss (Hennig, 1958). This method has been fully described in a previous paper (Dunnill, 1962). Five hundred points were counted on each slide, using the low power of the microscope, and these were found to cover the entire section.

Number of alveoli The method used was that described by Weibel and Gomez (1962 a). In the normal lung, the alveolar transections were counted in 10 fields on each slide. The fields were outlined by a square grating drawn on an eyepiece graticule. In the normal lung the area of the square was $9{\cdot}08 \times 10^{-3}$ cm.^{2} In the abnormal lung, a slightly different graticule was used with an area of $9{\cdot}13 \times 10^{-3}$ cm.^{2} The method is fully described in the original paper. The number of alveoli, N, in a unit volume is given by

$$N = \frac{n^{3/2}}{\beta\sqrt{\rho}} \qquad (1)$$

where n is the number of alveolar transections in a unit area, ρ is the volume proportion of alveoli, and β is a shape constant which for normal alveoli was estimated by Weibel and Gomez to have a value of 1·55.

Alveolar surface area The mean linear intercept method of Campbell and Tomkeieff (1952) and Hennig (1956) was employed. Crossed hair lines, each measuring 0·235 cm., were placed on 10 fields on each section of both normal and abnormal lungs, and the number of intercepts with respiratory tissue was counted. The mean linear intercept, L_m, was then calculated from the number of intercepts, m, the length of the line, 0·235 cm., and the number of times the line was placed on the sections, N, from

$$L_m = \frac{0.235N}{m} \qquad (2)$$

The alveolar surface area, S, then follows from

$$S = \frac{4V}{L_m} \qquad (3)$$

where V is the volume of the processed lung parenchyma. This value for S has then to be multiplied by the factor converting processed areas to fresh areas, p^2f^2, which in both these cases was 1·82.

SELECTION OF MICROSCOPIC FIELDS FOR HISTOLOGICAL ANALYSIS The selection of fields in which to count the number of alveolar transections or the number of intercepts presents a problem similar to that of the selection of blocks on the gross specimen. In order to obtain a representative and unbiased selection of fields, a random sampling procedure was adopted. The vertical and horizontal micrometer gauges on the mechanical stage of the microscope were employed together with a table of three digit random numbers. These numbers were used to give references on the horizontal and vertical gauges, and by this method 10 fields were easily selected.

RESULTS AND DISCUSSION

The estimates for the number of alveoli and for the alveolar surface area, in both the normal and the abnormal lung, are given in Table I. It is important to establish (1) that there is a significant variation between slides with regard to the number of alveolar transections and number of intercepts; (2) that there is a significant variation between slides, and between fields within slides, with regard to the volume proportions; (3) the standard errors of the volume proportions, the number of alveolar transections and the number of intercepts;

TABLE I

SUMMARY OF DATA ON NORMAL AND ABNORMAL LUNGS

	Normal Lung	Abnormal Lung
Volume of fresh lung at T.L.C. (ml.)	6,450	4,825
Volume of fixed lung	3,550	2,650
Conversion factor for fixed to fresh tissue (volume)	1·82	1·82
Percentage volume of non-parenchyma	7·75	7·00
Conversion factor for fixed to processed tissue (volume)	0·75	0·75
Volume of processed parenchyma (ml.)	2,463	1,848
Conversion factor for processed to fresh tissue (area)	1·82	1·82
Total number of alveoli	$286{\cdot}10^6$	$94{\cdot}10^6$
Total surface area (m.^{2})	75·8	39·1
Volume proportions (percentages)		
Alveolar air	55·64	40·84
Respiratory duct air	36·83	15.30
Abnormal air space air	—	30·49
Tissue	6·29	11·49
Vessels	1·24	1·88
Mean no. of alveolar transections per field	23·810	12·482
Mean no. of intercepts per field	19·875	13·690

and (4) what the standard error would be if the number of samples were smaller, as clearly it would be a great advantage if a reasonable estimate of these components of lung structure could be made on relatively few samples of tissue.

A model has been used where the observation in the j^{th} field of the i^{th} slide is given by

$$y_{ij}=\mu+a_i+e_{ij} \quad (i=1,\ldots.I;\ j=1,\ldots J) \quad (4)$$

where the sets a_i and e_{ij} are independent random variables with zero means. The a_is are identically distributed with variance σ_s^2, the e_{ij}s are identically distributed with variance σ_e^2; a_i is thus the component of variance between slides and e_{ij} the residual variance. For the significance tests the a_is and the e_{ij}s are assumed to be normally distributed. This will give rise to the following component variance analysis.

Source	Degrees of Freedom	Sum of Squares	Estimated Mean Square
Between slides	I - 1	$\sum_{i=1}^{I} \frac{Y_{i.}^2}{J} - \frac{Y_{..}^2}{IJ}$	$\sigma_e^2 + J\sigma_s^2$
Residue ..	I(J-1)	$\Sigma y_{ij}^2 - \frac{\Sigma Y_{i.}^2}{J}$	σ_e^2
Total	IJ-1	$\Sigma y_{ij}^2 - \frac{Y_{..}^2}{IJ}$	

The standard error, $\sigma_{\bar{y}}$, of the population mean, $\bar{y}$, will be given by

$$\sigma_{\bar{y}}=\sqrt{\frac{1}{IJ}(\sigma_e^2+J\sigma_s^2)} \quad (5)$$

This relationship can be used to determine the standard error of the mean in any experiment where I blocks of tissue (slides) are taken and J fields counted on each slide.

In the normal lung, the differences between slides are unlikely to be very great due to the uniformity of parenchymal structure in the normal organ. The analysis of variance with respect to the number of alveolar transections illustrates this point.

Source	Degrees of Freedom	Sum of Squares	Mean Square
Between slides	19	148·38	7·809
Residue	180	2890·40	16·058
Total ..	199	3038·78	

This gives a value for Residual mean square/Between slides mean square of 2·056. This seems to indicate that the differences between slides are less than those between fields within slides. However, since the significance level is only that of 5%, or 1 in 20, no great importance should be placed on this result. This means that there is no significant difference between slides. In this case, as the values for the residual mean square exceed the between slides mean square, the larger mean square has been used to estimate the standard error of the mean. Equation (5) then becomes

$$\sigma_{\bar{y}}=\sqrt{\frac{1}{IJ}\sigma_e^2}$$

The estimate of the population mean of alveoli per field is 23·81 with a standard error of 0·28, or approximately 1·2%. Clearly, in the normal lung, a far smaller number of blocks would give an adequate estimate. It can be seen that with five blocks of tissue (slides) and 10 fields per slide the standard error would still be only of the order of 2%.

In the case of the number of intercepts per field in the normal lung, the analysis of variance is given below, showing that there is no significant difference between slides. The value for Residual

Source	Degrees of Freedom	Sum of Squares	Mean Square
Between slides	19	167·8	8·83
Residue ..	180	1,960·1	10·89
Total ..	199	2,127·9	

mean square/Between slides mean square is 1·233, which is not significant. The estimate of the mean number of intercepts per field is 19·875 with a standard error of 0·233. Five slides and 10 fields per slide would give an error of 0·467 or 2·3%.

In the emphysematous lung there is a much greater difference between slides, as is shown by the analysis of variance for alveolar transections.

Source	Degrees of Freedom	Sum of Squares	Mean Square
Between slides	39	3,559·18	91·261
Residue ..	360	16,903·70	46·955
Total ..	399	20,462·88	

This gives a value for F of 1·94, which shows that there is a significant difference between slides at the 0·5% level. The mean number of alveolar transections per field is 12·482 and, using equation (5), the standard error is 0·470 or approximately

3·8%. If, say, 10 slides were chosen, then the error would be 0·95 or 7·6%.

A similar analysis of variance for the number of intercepts per field is given below.

Source	Degrees of Freedom	Sum of Squares	Mean Square
Between slides	39	1,709·96	43·845
Residue ..	360	9,174·60	25·485
Total ..	399	10,884·56	

This gives an F value of 1·72, which shows there is a significant difference between slides at the 1% level. The standard error of the mean number of intercepts per field, 13·69, is 0·331 or 2·4%. If 10 fields on each of 10 slides were sampled, this would give a standard error of 0·66 or 4·8%.

The greater error found when dealing with the numbers of alveoli, as opposed to the numbers of intercepts, is due to the great variation in shape and size of the normal alveoli in an emphysematous lung and also to the difficulty of identifying, with certainty, all the normal alveoli in a given area on a given section of lung.

An analysis of variance can be applied to the estimate of the volume proportions. In the normal lung the differences within slides and between slides are not significant. In the emphysematous lung this is not so. In this case, 20,000 points were counted on 40 slides. The position of each point was assessed, and the points were recorded in fields of 100 points. Thus, every 100 points, the number lying in each of the components, alveolar air, alveolar duct air, abnormal air space air, tissue, and vessels was listed. There were five fields for each slide. The analysis of variance for alveolar air, alveolar duct air, and abnormal air space air is given in Tables II, III, and IV.

TABLE II

ANALYSIS OF VARIANCE FOR ALVEOLAR AIR IN EMPHYSEMATOUS LUNG

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Between slides ..	39	114·268	2·930	4·47
Between fields (within slides) ..	160	107·288	0·6706	2·88
Residue ..	19,800	4,610·450	0·23285	
Total ..	19,999	4,832·006		

The F values show that the variance between slides, and between fields within slides, are both significant at the 0·1% level.
The estimate of the proportion is 0·40835 with a standard error of 0·0121.

$\sigma_e^2 = 0{\cdot}23285$
$\sigma_f^2 = 0{\cdot}00438$
$\sigma_s^2 = 0{\cdot}00452$

TABLE III

ANALYSIS OF VARIANCE FOR ALVEOLAR DUCT AIR IN EMPHYSEMATOUS LUNG

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Between slides ..	39	33·196	0·8512	3·401
Between fields (within slides) ..	160	40·048	0·2503	1·967
Residue ..	19,800	2,519·270	0·1272	
Total ..	19,999	2,592·514		

The F values show that the differences between slides, and between fields within slides, are both significant at the 0·1% level.
The estimate of the proportion is 0·15305 and the standard error is 0·0065.

$\sigma_e^2 = 0{\cdot}12723$
$\sigma_f^2 = 0{\cdot}00123$
$\sigma_s^2 = 0{\cdot}00120$

TABLE IV

ANALYSIS OF VARIANCE FOR ABNORMAL AIR SPACE AIR

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Between slides ..	39	230·7638	5·9170	5·40
Between fields (within slides) ..	160	175·2760	1·0955	5·66
Residue ..	19,800	3,832·68	0·19357	
Total ..	19,999	4,238·7198		

The F values show that the variance between slides, and between fields within slides, are both significant at the 0·1% level.
The estimate of the proportion is 0·3049 with a standard error of 0·0172.

$\sigma_e^2 = 0{\cdot}19357$
$\sigma_f^2 = 0{\cdot}00902$
$\sigma_s^2 = 0{\cdot}00964$

A model for the analysis of variance for proportions of lung tissue was used where the observation of the k^{th} point in the j^{th} field of the i^{th} slide was given by

$$y_{ijk} = \mu + a_i + b_{ij} + e_{ijk} \qquad (6)$$
$$(i=1, \ldots . I_j \quad j=1, \ldots . J \quad k=1, \ldots . K)$$

were μ is the overall mean, a_i is the between slides effect, b_{ij} is the between fields within slides effect, and e_{ijk} is the residual term. y=1 when the point lies in the component being estimated, *e.g.*, alveolar air, duct air, etc., and y=0 otherwise. The a_is, b_{ij}s, and e_{ijk}s are all independently distributed with zero means; the a_is identically with variance σ_s^2, the b_{ij} s identically with variance σ_F^2, and the e_{ijk}s identically with variance σ_e^2. For the significance tests these variates are also

TABLE V

Source	Degrees of Freedom	Sum of Squares	Expected Mean Square
Between slides	I - 1	$\sum_{i=1}^{I} \frac{Y_{i..}}{JK} - \frac{Y_{...}^2}{IJK}$	$\sigma_e^2+K\sigma_F^2+JK\sigma_s^2$
Between fields (within slides)	I(J - 1)	$\sum_{i=1}^{I}\sum_{j=1}^{J} \frac{Y_{ij.}^2}{K} - \sum_{i=1}^{I} \frac{Y_{i..}^2}{JK}$	$\sigma_e^2+K\sigma_F^2$
Residue	IJ(K - 1)	$\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K} y_{ijk}^2 - \sum_{i=1}^{I}\sum_{j=1}^{J} \frac{Y_{ij.}^2}{K}$	σ_e^2
Total	IJK - 1	$\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K} y_{ijk}^2 - \frac{Y_{...}^2}{IJK}$	

assumed to be normally distributed. The model component variance is shown in Table V. In such a model with a random sample of I slides, J fields per slide, and K points per field, the standard error, σ_ρ, of the volume proportion, ρ, is given by

$$\sigma_\rho = \sqrt{\frac{1}{IJK}(\sigma_e^2+K\sigma_F^2+JK\sigma_s^2)} \qquad (7)$$

Thus it is possible to calculate the standard error of the volume proportions of the various components if, say, only 10 blocks of tissue had been selected. Assuming 100 points were counted per field, and there were five fields per slide, the standard errors for the proportions of alveolar air, duct air, and abnormal air space air would be 0·024, 0·013, and 0·034 respectively.

It can be seen that with as few as five blocks of tissue in the normal lung and 10 blocks in the abnormal lung a fairly accurate estimate of quantitative anatomical data can be obtained. In the normal lung the final values for the number of alveoli and for the alveolar surface area are in good agreement with those of Weibel and Gomez (1962 b). The figures for the number of alveolar transections and for the number of intercepts, together with their corresponding analyses of variance, illustrate well the uniformity of the normal lung parenchyma.

In the emphysematous lung the mean linear intercept method is particularly reliable when used with this method of sampling. This is because it is independent of the size, shape, and configuration of the structures whose total surface area is being estimated. The greater variation in the estimation of the numbers of alveoli in this lung is due to a combination of factors. Probably the most important of these is the variation in shape of the remaining normal alveoli in this disease. In those areas adjacent to the centrilobular spaces, the normal alveoli may be distorted or different in size from those present in normal lung.

In deciding the number of blocks to be taken in any given case, the construction of a summation average graph may be of great assistance. This entails calculating the mean of, say, the number of alveolar transections after each field has been counted. One such graph for a normal lung is illustrated in Fig. 1. It can be seen that for the first few observations the mean oscillates considerably, but that after a number of observations, in this case 50, corresponding to five slides, the mean remains steady within very narrow limits. In emphysematous cases the mean takes longer to reach its steady value. When this steady state has been achieved, there is little point in continuing the observations as these will not alter the mean significantly.

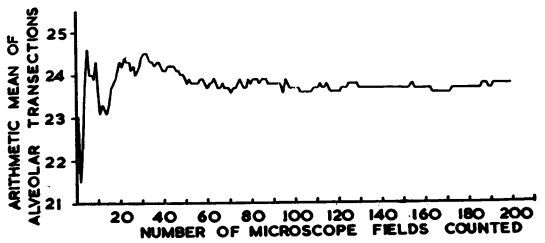

FIG. 1. *A summation average graph plotted for the alveolar transections of the normal lung. It can be seen that after 50 fields have been counted, i.e., five slides, the mean varies very little, in fact less than* 0·3.

The results shown here indicate that it is possible to obtain reliable quantitative data in pathological lungs with relatively few blocks of suitably selected and prepared tissue. This fact is likely to be of considerable importance in the correlation of pathology with respiratory function tests performed during life. In the future it is hoped that similar methods of sampling may be used in the quantitative morphological analysis of other organs.

SUMMARY

A simple method for sampling the lung for quantitative morphological analysis is described and evaluated. It employs the principle of random sampling and gives a representative picture of

pathological lungs with the selection of relatively few blocks of tissue. Two examples are given, one a normal lung and the other a lung from a case of diffuse centrilobular emphysema.

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