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MORPHOMETRY OF NON-RANDOM STRUCTURES RELATED TO OXYGEN FLOW IN THE MAMMALIAN RESPIRATORY SYSTEM

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Abstract. Biological structures are part of a highly organized non-random system. Problems arise when morphometric studies are performed because stereological methods depend on "randomness" between structure and measuring probe. This can be overcome by suitable models and sampling strategies, as is shown on the respiratory system of mammals. Three cases of non-randomness are considered in their methodological and physiological implications: (1) the distribution of mitochondria in muscle cells shows a gradient from the periphery to the center; (2) the capillaries of muscle fibers are anisotropic, and may show a density gradient from arterial to venous end; (3) the gas exchange units of the lung are non-randomly arranged on a branched airway tree whose properties can be described by fractal geometry.

INTRODUCTION: THE DILEMMA OF MORPHOMETRY

By their very nature all biological structures are non-random. They come about by orderly morphogenetic processes which place the elements into precise relationships. A high degree of spatial order is, in fact, required for the execution of most bodily functions. The examples are rare where one could say that randomness is the governing principle of biological structure.

On the other hand, the most powerful methods for obtaining morphometric information, namely those of stereology, require stochastic conditions—that is random interactions between the measuring probes and the structures under study—because they are based on principles of geometric probability (Weibel: 1979, 1980). We are hence confronted with the dilemma of how to obtain reliable and sensible information on the non-random biological structures by yet making use of the potential offered by stereological methods, particularly because non-stereological methods of morphometry are usually much less efficient, and often biased.

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The solution to this dilemma is to begin the morphometric study with a careful descriptive investigation of the structural system under consideration, combined with an analysis of the functional implications of structural design. This may lead to model concepts on how the system works, preferably formalized in a set of equations, and it will allow us to sort out those parameters which depend on structural design, for which we need morphometric information. By looking at these structures one by one—but never forgetting their position in the system—we have considerably reduced the degree of non-randomness and can now often use stereological methods, introducing randomness through appropriate sampling strategies (Weibel: 1979; Cruz-Orive & Weibel: 1981). Some advanced methods of stereological sampling will even allow us to use some aspects of non-random structure to our advantage, such as the so-called vertical sections (Baddeley *et al.*: 1986), the Cavalieri principle (Michel & Cruz-Orive: 1988), or the disector methods (Sterio: 1984; Cruz-Orive: 1987). The results of such studies receive their full meaning only if they can be introduced into the model of the system, as we shall discuss for the respiratory system.

However, there will often remain some problems which cannot be reduced to the conditions where stereological methods become applicable. This will require special approaches and solutions, some of which will be discussed.

A MODEL CASE: THE MAMMALIAN RESPIRATORY SYSTEM

The supply of O_2 to muscle cells is a vital function needed to allow the production of energy by combustion of substrates. At rest a human consumes about 250 ml O_2 each minute, but in heavy exercise this rate increases to 2.5 liters per minute in a healthy individual, or to up to twice as much in a well-trained elite athlete. Oxygen is collected from environmental air in the lung, transferred to blood, transported to the muscles by circulation, discharged to the cells from the capillaries, and eventually consumed in the mitochondria in the process of oxidative phosphorylation. Figure 1 shows this pathway for O_2 in a simple model. For each level we have formulated the flow rate $\dot{V}_{O_2}$ as function of a driving force multiplied

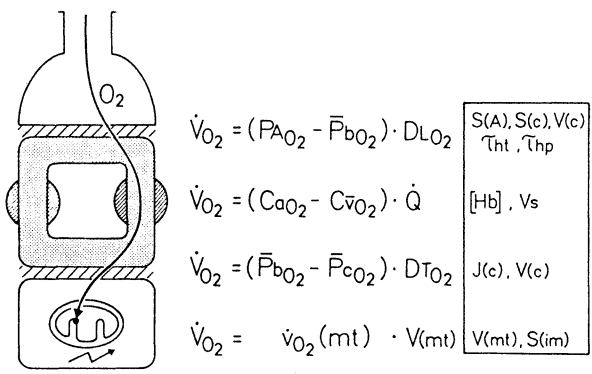


Fig. 1. Model of respiratory system.

with a conductance parameter. The driving forces are differences between physiological variables, whereas the conductance terms are determined, to an important degree, by the morphometric parameters which are listed in the box. Thus we see that the diffusing capacity of the lung, DL_{O_2}, is determined by the gas exchange surface area, SA, the capillary blood volume, Vc, and the harmonic mean thickness of the air-blood tissue barrier, τ_h, which must be introduced into a set of equations to calculate DL_{O_2} (Weibel: 1970/71). Similar parameters determine the diffusing capacity of the capillary network in the muscle, but one parameter stands out, the length of capillaries, $J(c)$, from which volume and surface of the capillary tubes follow. Finally, we find that the rate at which muscle cells consume O_2 is related to the volume of mitochondria they contain, and particularly to the surface area of inner mitochondrial membranes which house the oxidase system as well as the enzymes performing the synthesis of ATP, the cell's essential fuel.

The study of structure-function relationship in this respiratory system requires the estimation of these morphometric parameters in conjunction with well controlled physiological measurements. We have chosen an approach where $\dot{V}_{O_2}$ varies between different animals in a well determined way (Weibel & Taylor: 1981; Taylor *et al.*: 1987) so that we can sort out the importance of structural and functional adaptations to different levels of O_2 flow rate, taking the maximal level of O_2 consumption, $\dot{V}_{O_2}max$, as a standard reference parameter for the system, because under limiting conditions the system is used to its full extent and "weak links" in the chain should become apparent. From a our point of view the first question is how large the different morphometric parameters must be to allow the flow of O_2 at the maximal rate measured, in order to find out to what extent structure contributes to adaptation and to limitation of O_2 flow rate.

But we have gone one step further and have postulated that the structures at the different levels must be linked quantitatively, taking the non-randomness of the system into consideration. We have formulated this in the hypothesis of *symmorphosis* which states that the design of each part of the system should be quantitatively—morphometrically—matched to that of all others, and have developed a strategy to test this hypothesis (Weibel *et al.*: 1987a).

From a morphometric point of view this study is straightforward: all the parameters identified in Fig. 1 can be measured by stereological methods, at least in first approximation. Figures 2 and 3 show electron micrographs of lung and muscle tissue which reveal that the structures of the pulmonary gas exchanger as well as the mitochondria in muscle cells are sufficiently "randomly" distributed to allow their measurement by classical methods of stereology, if only we take care to properly sample the tissue to avoid bias.

This approach has recently been used in a study of adaptive variation of the respiratory system by comparing "athletic" species, such as dogs and horses, to "normal" species of the same size class, goats and cows. The athletic species achieve a $\dot{V}_{O_2}max$ which is 2.5 times greater, and the question was whether the various morphometric parameters were increased proportionately at all levels of the system (Taylor *et al.*: 1987). The salient results are shown in condensed form in Tables 1–3. For the mitochondria we find that their total volume is strictly proportional to $\dot{V}_{O_2}max$ (Table 1) with the result that the unit volume of muscle mitochondria

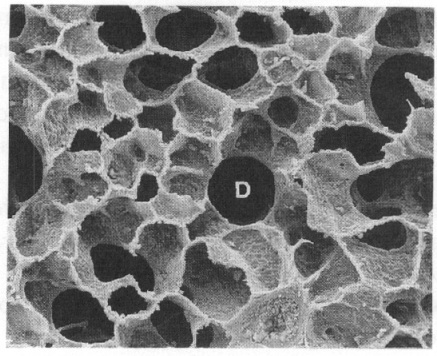


Fig. 2. Scanning electron micrograph of human lung parenchyma with alveoli densely packed around alveolar duct (D).

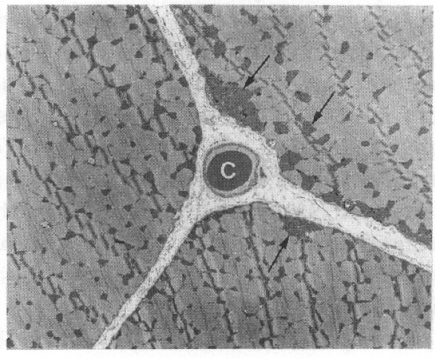


Fig. 3. Three muscle fibers surrounding capillary (C) contain mitochondria (arrows) throughout cell.

Table 1. Factors by which $\dot{V}_{O_2}$max, total mitochondrial volume V(mt), and mitochondrial O_2 consumption rate $\dot{V}_{O_2}$(mt) are increased or "adapted" in three athletic species (dog, pony, horse) over non-athletic species.

	O_2 consumption by mitochondria $\dot{V}_{O_2}\text{max} = V(\text{mt}) \times \dot{V}_{O_2}(\text{mt})$		
Dog/Goat	2.4	2.9	(0.8)
Pony/Calf	2.4	2.2	(1.1)
Horse/Steer	2.6	2.5	(1.0)

consumes a constant amount of O_2 at $\dot{V}_{O_2}$max (Hoppeler *et al.*: 1987), a rule that is bourne out by several studies on different adaptive situations. With respect to microcirculation we find that the capillary length is only partially adapted to $\dot{V}_{O_2}$max (Table 2); but it is most interesting that in the athletic species the hematocrit, the volume density of erythrocytes in the blood (another morphometric parameter) is also increased, with the result that the total volume of erythrocytes

Table 2. The tissue diffusing capacity, DT_{O_2} is adapted to $\dot{V}_{O_2}max$ by combined increase in capillary volume, $V(c)$, and hemoglobin concentration, [Hb].

O_2 delivery by microcirculation $\dot{V}_{O_2}max = DT_{O_2} \times (\bar{P}b_{O_2} - \bar{P}c_{O_2})$				
		$V(c)$	[Hb]	
Dog/Goat	2.4	1.8	1.7	(0.8)
Pony/Calf	2.4	1.6	1.7	(0.9)
Horse/Steer	2.6	1.5	1.4	(1.2)

Table 3. The pulmonary diffusing capacity, DL_{O_2}, is only partially adapted to $\dot{V}_{O_2}max$.

O_2 uptake by lung $\dot{V}_{O_2}max = DL_{O_2} \times (\bar{P}A_{O_2} - \bar{P}b_{O_2})$			
Dog/Goat	2.4	1.5	(1.6)
Pony/Calf	2.4	1.6	(1.5)
Horse/Steer	2.6	2.0	(1.3)

available for delivering O_2 to the cells is proportional to $\dot{V}_{O_2}max$ (Conley *et al.*: 1987). Whereas in both these cases the higher $\dot{V}_{O_2}max$ is matched by structural adaptations, even though two structures (capillaries and erythrocytes) may have been involved, the lung fails to adjust its structures to match the higher need for O_2 uptake in the athletic species. As Table 3 shows, the diffusing capacity is increased only by about 50% so that the higher uptake rate must be achieved by functional adaptations, augmenting the driving force across the air-blood barrier (Weibel *et al.*: 1987b).

In terms of the hypothesis of symmorphosis, which we set out to test with these experiments, we conclude that the structural design of the system is matched to functional demand at the level of the muscle cells and their microvasculature, but that it fails to be matched in the lung. We shall see that this may partly be due to an inordinate degree of simplification in our structural model of the pulmonary gas exchanger, in that we have not adequately accounted for non-randomness in the distribution of gas exchange unit with respect to airways and blood vessels.

THE IMPORTANCE OF STRUCTURAL NON-RANDOMNESS

At each of the three levels of the respiratory system considered in the preceding section the structures involved had non-random features. With respect to the model used (Fig. 1), however, they appeared irrelevant, at least in first approximation. We therefore used sampling strategies which eliminated effects of non-randomness from the results. However, if one looks in greater depth at some of the physiological processes involved one may need to account for the precise spatial relationship between the structural elements. Examples for this are models of microvascular gas exchange in muscle where the respective orientation of capillaries and muscle fibers, as well as the distribution of mitochondria are of importance. Another case is the

modelling of ventilation processes in the lung; this involves a characterization of the branching pattern of airways, where stereological methods fail.

NON-RANDOM DISTRIBUTION OF MUSCLE MITOCHONDRIA

It has long been known that muscle cells have mitochondria principally in two locations: interfibrillar mitochondria are interspersed between the myofibrils throughout the muscle cell, whereas subsarcolemmal mitochondria are found in the marginal cytoplasm between the sarcolemma and the outermost myofibrils. One important question is whether these are two different classes of mitochondria (Palmer *et al.*: 1977), or whether they merely represent different locations of the same type of organelle.

This question cannot be answered by limiting the observation to random sections of muscle, as they are required for stereology. It is a topological question, and this necessitates the study of structure in three dimensions, because single sections do not permit topological parameters, such as number or connectivity, to be measured. To a certain extent this question could be approached by using the disector (Sterio: 1984) or the selector method (Cruz-Orive: 1987), which employ pairs of sections. The most complete information is however obtained by reconstruction from serial sections (Kayar *et al.*: 1988b). This type of study has revealed a high degree of structural complexity of mitochondria in muscle cells (Fig. 4): some mitochondria form extensive networks that extend from the subsarcolemmal region into the depth of the muscle fiber. This suggests that subsarcolemmal and interfibrillar mitochondria may be one and the same structure. It is also seen that mitochondria show preferred orientations: they preferentially form reticula in the cross section plane of the fiber in the region of the I-bands, but then show straight segments that are oriented parallel to the myofibrils. It is quite evident that mitochondria are non-randomly arranged within the muscle cells.

When studying different muscle types one finds, as a general rule, that mitochondria are systematically distributed with respect to the muscle cross section in the sense that they are more concentrated towards the fiber periphery, as can be seen in Fig. 3. Kayar *et al.* (1988a) have looked closer at this distribution and have found that the mitochondrial concentration is highest near the capillaries and decreases systematically towards the fiber center (Fig. 5); the gradient is found to be very similar for different muscle types (Fig. 6). We shall examine the importance of

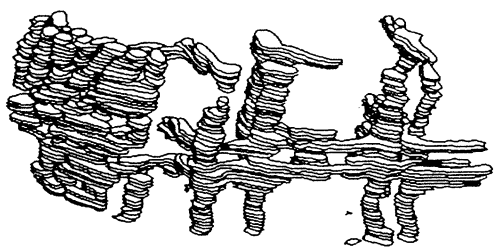
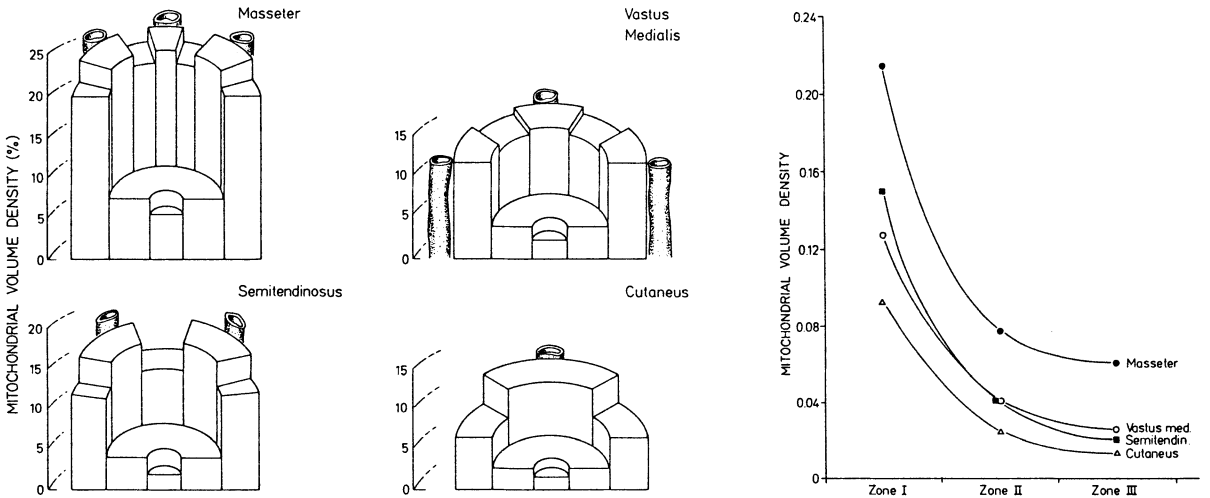


Fig. 4. Reconstruction of one mitochondrion from a muscle fiber shows high degree of complexity.



Figs. 5 and 6. Distribution of mitochondrial volume density showing the gradient from cell periphery to core of muscle fiber, and particulaly high density near the capillaries. Note similar distribution in four muscles. (From Kayar *et al.*: 1988)

this gradient below when discussing new models for O_2 delivery from the capillary to the mitochondria. The method used to obtain these distributions was based on the estimation of local volume density of mitochondria by sampling the muscle fibers non-randomly, recording the location of each field sampled with respect to the muscle fiber cross section (Kayar *et al.*: 1988a).

NON-RANDOM STRUCTURE OF CAPILLARY NETWORK IN MUSCLE

It is well known that the form of capillary networks is fitted to the shape of the tissue structures they supply. Accordingly, muscle capillaries will be arranged more or less parallel to the muscle fibers. This property guided August Krogh (1919) when he designed a model for O_2 supply to muscle cells, taking the capillaries to be straight tubes of a certain length surrounded by a cylindrical sleeve of muscle cell mass, what is now called a "Krogh cylinder" (Fig. 7). The critical parameters of the Krogh cylinder are its length, estimated at about 0.5–1 mm, and its radius R_k which was derived from the "capillary density", i.e. the number of capillary profiles found per mm^2 of muscle cross section. With a capillary density of about 400 mm^{-2}, a typical value for a human leg muscle, one finds $R_k \approx 28 \mu m$. By considering the change in P_{O_2} of capillary blood, as it moves from arteriole to venule and delivers O_2 to the cells, Krogh calculated that a cylinder of this radius should just be right to avoid anoxic regions to occur near the venous end of the capillary path.

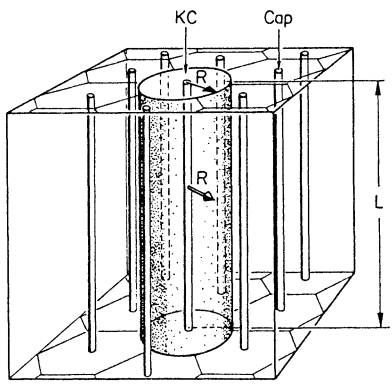


Fig. 7. Krogh cylinder model. (From Weibel: 1984)

The first improvement to be introduced is to examine the true degree of preferred orientation of muscle capillaries (Mathieu *et al.*: 1983). In the model of the respiratory system (Fig. 1) we have used the total length $J(c)$ as the basic measure for the capillary network. The basic morphometric parameter is the length density per unit volume of muscle (or muscle fiber), $J_V(c, f)$, which is related to the number of capillaries counted on the unit area of muscle cross section, the "capillary density" of Krogh, $N_A(c, f)$. The relationship depends, however, on the degree of orientation or anisotropy of the capillaries (Fig. 8): If the capillaries are straight parallel tubes

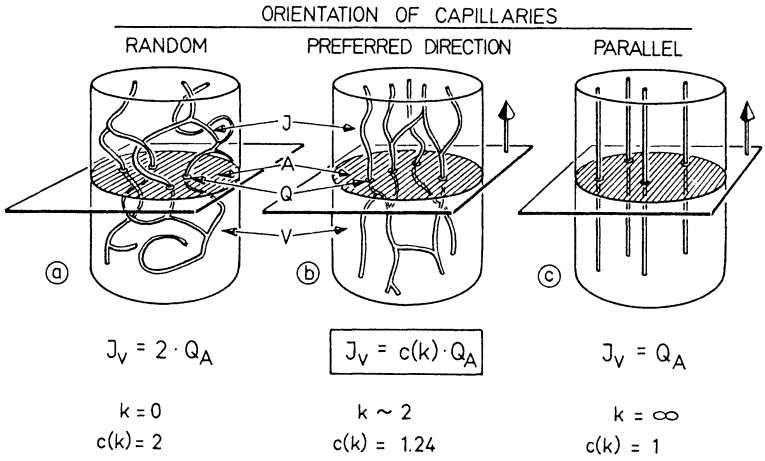


Fig. 8. Effect of orientation on relation between "capillary density" $N_A = Q_A$ and length density J_V. (From Weibel: 1984)

we have

$$J_V(c, f) = N_A(c, f) \tag{1}$$

the relation used by Krogh; but if they form random networks we have

$$J_V(c, f) = 2 \cdot N_A(c, f) \tag{2}$$

the standard stereological equation for length density (Weibel: 1979). For the general case of preferred orientation with respect to an orientation axis, such as the direction of muscle fibers, we can write

$$J_V(c, f) = c(K, 0) \cdot N_A(c, f) \tag{3}$$

where $c(K, 0)$ is a coefficient of orientation whose value must lie between 1 for total anisotropy and 2 for random orientation. The parameter K is the concentration parameter for the Dimroth-Watson orientation distribution, and 0 relates to the use of cross sections whose normal is parallel to the orientation axis (Cruz-Orive *et al.*: 1985).

It is evident from equations (1)–(3) that the simple use of "capillary density" $N_A(c, f)$ in model calculations of the Krogh cylinder type may introduce errors of up to a factor of 2, since, in fact, the Krogh cylinder model should be based on the length density of the capillary (Weibel: 1984). It is therefore important to assess the degree of preferred orientation of capillaries and to estimate the value of the coefficient $c(K, 0)$ (Mathieu *et al.*: 1983). For this purpose we first must establish that the Dimroth-Watson distribution function is valid for muscle capillaries; Figure 9 shows that this is the case, as the values of $N_A(c)$ obtained at different angles of

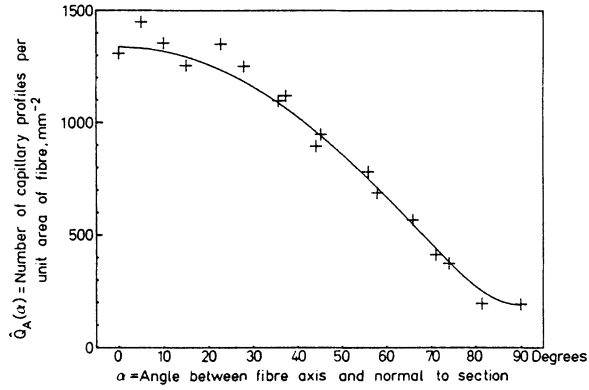


Fig. 9. The number of capillary profiles (crosses) counted on sections cut at different angles to the muscle fiber axis is well described by Dimroth-Watson distribution function (curve). (From Cruz-Orive *et al.*: 1985)

sectioning are well described by this distribution function (Cruz-Orive *et al.*: 1985). Comparing different muscles it was then found that $c(K, 0)$ was ~ 1.07 for heart and ~ 1.24 for skeletal muscles (Conley *et al.*: 1987). Mathieu-Costello (1987) furthermore showed that $c(K, 0)$ varied with the degree of contraction at which the muscle was fixed, so that it could be expressed as a function of sarcomere length. Using equation (3) we can now estimate realistic values of capillary length density in various muscles under different conditions, and then use Krogh-type models to assess O_2 delivery to muscle cells and their mitochondria.

There is, however, one further point to be noted. It appears that the density of capillaries is not uniform along the capillary path: it has been noted that capillaries become denser as they approach the venous end, so that the "Krogh cylinder" should actually be conceived as a "Krogh cone" which tapers towards the venous end, as shown in Fig. 10 (Weibel: 1984). This has important implications on the functional interpretation of O_2 delivery to the cells: the diameter of the cone appears matched to the P_{O_2} profile in the capillary which falls towards the venous end so that the driving pressure for O_2 diffusion into the cell is reduced. But in this region the blood needs to serve a smaller cross section.

Let us now consider that the P_{O_2} within the muscle cell also shows a profile, being highest near the capillary and falling towards the center of the fiber as O_2 is consumed by the mitochondria. We have shown in Figs. 5 and 6 that the mitochondria are inhomogeneously distributed on the muscle cross section, their density being highest near the capillary and falling towards the center. It is therefore highly suggestive that the distribution of mitochondria is also matched to the P_{O_2} profile, so that local O_2 consumption is uneven throughout the cell, being highest where O_2 supply is best. This type of information will have to be considered in future model calculations on muscle O_2 consumption and energy supply to myofibrils.

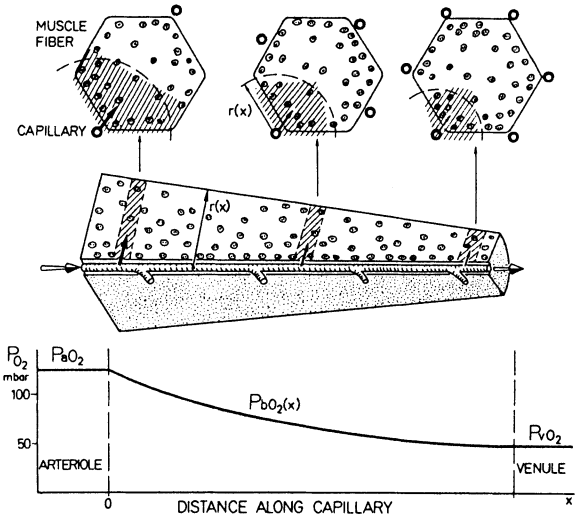


Fig. 10. Shape of "Krogh cone" appears matched to P_{O_2} profile in capillary. (From Weibel *et al.*: 1981)

NON-RANDOM DISTRIBUTION OF GAS EXCHANGE UNITS IN THE LUNG

We have noted in Table 3 that the pulmonary diffusing capacity does not appear to be well matched to the required O_2 uptake rate, and have remarked that this finding may partly be due to an inordinate simplification of the model used to calculate diffusing capacity, particularly that we may have not sufficiently accounted for non-random arrangement of the gas exchange units with respect to airways and blood vessels, thus disregarding effects of ventilation and perfusion.

The efficiency of gas exchange in the lung, indeed, crucially depends on a good match between the ventilation and the perfusion of the about 300 million gas exchange units. These units are arranged along the branched system of alveolar ducts (Fig. 11) which constitute the 9–10 last generations of the bronchial tree and form the acinus (Haefeli-Bleuer & Weibel: 1988). In terms of ventilation all gas exchange units within one acinus form a unit: they are ventilated through a common port, and are arranged in series along intraacinar airways. In terms of perfusion, however, each gas exchange unit is independent as each is perfused directly from a terminal branch of the pulmonary artery (Fig. 13). This may have an important effect on the functioning of the gas exchange units. Since the acinar airways are ventilated mostly by diffusion of O_2 in the alveolar duct, and since the gas exchange units take up O_2 from the air, one must postulate the existence of a P_{O_2} gradient along the alveolar ducts so that the last alveoli are less well ventilated than those near the entrance into the acinus (Fig. 13). We have therefore postulated that the efficiency of gas exchange could be reduced due to unmatched ventilation and perfusion of the units, and that this would depend on the morphometric properties of the acinus (Weibel *et al.*: 1981).

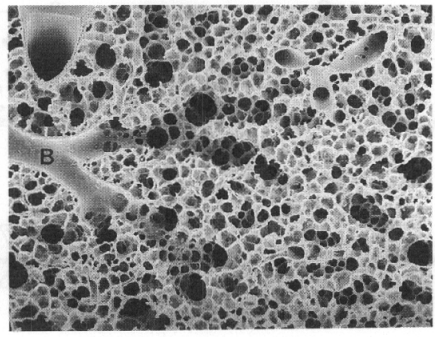


Fig. 11. Terminal bronchiole (B) branches into lung parenchyma as alveolar ducts densely surrounded by alveoli.

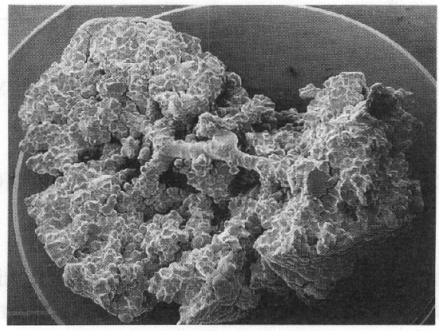


Fig. 12. Cast of human pulmonary acinus shows branching of intraacinar airways. (From Haefeli-Bleuer & Weibel: 1988)

To study these non-random properties of acinar airways is not easy and cannot depend on stereological methods, because it must record all the measurements with respect to their location in the hierarchy of the branched airway tree. We have therefore resorted to a detailed morphometric study of acinar airways using silicon rubber casts, as shown in Fig. 12, sampling airways by rigorous random sampling techniques, and then dissecting and measuring each branch (Rodriguez *et al.*: 1987; Haefeli-Bleuer & Weibel: 1988). We then found, for example, that in the human lung the intraacinar airways branch over about nine generations and that the total intraacinar pathlength averages 8 mm. Combined with morphometric data on the gas exchange units, obtained with stereological methods, we can now define precise models for intraacinar gas exchange which should shed light on the question of efficiency of gas exchange.

The last question relating to non-random structures in the lung relates to the design of the airways and blood vessels. To what extent does the design of the airway and vascular trees ensure matched ventilation and perfusion of the gas exchange units?

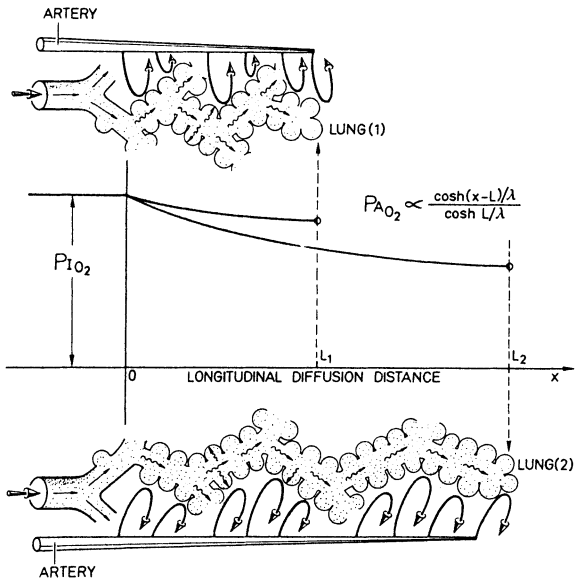


Fig. 13. P_{O_2} profile along branched alveolar ducts may be a function of acinar path length. (From Weibel *et al.*: 1981)

Bronchi and pulmonary artery form parallel branching trees. Both multiply their branches by dichotomy, strictly in parallel for about the first 10–14 generations. Beyond the 14th generation, on the average, the airways assume the nature of intraacinar airways with alveoli coating their wall (Fig. 11), and the arteries form supernumerary branches that supply adjacent gas exchange units. Branching is irregular, but it follows systematic rules. Thus, the dimensions of the branches decrease systematically with each generation, following a similar pattern for airways and arteries: on the average, one finds the diameter to be reduced by ~0.6, and the length by ~0.8. This means that branching is similar as we proceed from generation to generation; what changes is the scale. This opens the possibility to describe the bronchial and the vascular trees as fractal structures (Mandelbrot: 1982) and to exploit this powerful new geometry to explore the features of these trees that may ensure matching of ventilation and perfusion. The main point is that the trees are self-similar: when plotting the mean airway diameter against the generation on a log-log plot the points lie about a straight line (Fig. 14). This fractal nature of the trees means that terminals, e.g. entrance bronchioles to acini, are reached along similar paths despite the apparent irregularity of branching. Accordingly, the design of the trees ensures a basic homogeneity of ventilation and of perfusion of the units.

However, there is a source for potential inhomogeneity in this design. The airway dimensions do not follow the fractal regression line perfectly (Fig. 14). It has been shown that the variation also has a "fractal pattern" (West *et al.*: 1986); the pattern of variation is something like an imprint of a diffusion front. The effect of this "organized irregularity" on the potential inhomogeneity of ventilation (and perfusion) will have to be studied carefully.

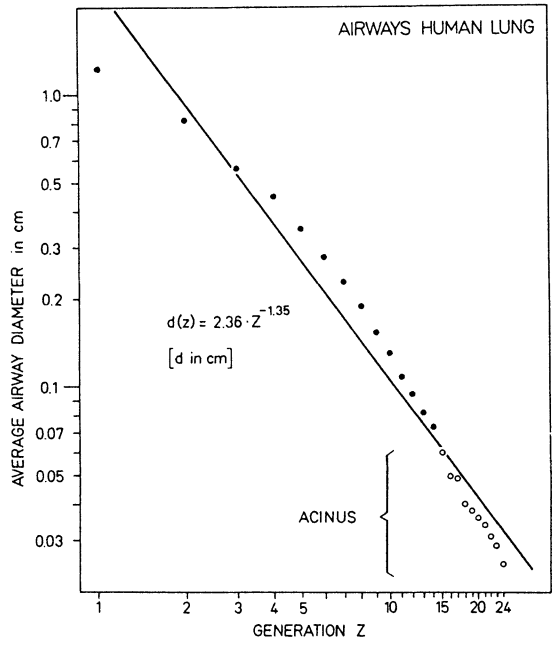


Fig. 14. Log-log plot of airway diameter against branching generation for human lung.

CONCLUSION

The dilemma of morphometry is real and not easily solved. By using randomization procedures one may well reduce even highly organized non-random structures to "near-random" conditions so that the powerful tools of stereology can be used to generate highly valuable data which can be used in studies on structure-function relationships. However, this approach hides functionally relevant features of non-randomness which can be uncovered only by searching for them and by using partly unconventional approaches.

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DISCUSSION

Q. Do you know which transfers or "diffuses" fastest, oxygen or ATP? Also, has the distribution of ATP been adapted to minimize the transfer time?

(Walton, O.)

A. The problem is not that easy. Muscle cells contain myoglobin which is an "O^2

buffer" but may also facilitate O^2 diffusion. The question is which is more important? In muscle cells with few mitochondria, i.e. larger diffusion distances, there is less myoglobin than in those with many mitochondria. ATP transfer from mitochondria to myofibrils is not simple. High energy phosphate bonds are "flowing" through a phosphocreatine shuttle which also serves as a high energy phosphate buffer. This shuttle may actually be faster than O^2 diffusion, but this needs to be worked out.

Q. You talked about structure (or non-randomness). It seemed to me that you called a structure non-random after you knew its function. Then, is it not possible to discriminate random and non-random of a given pattern without knowing its function? (Takaki, R.)

A. Yes, it is certainly possible to determine the degree of randomness or non-randomness by pattern analysis. For example, if structures are evenly distributed they are probably non-random, because random (Poisson) processes lead to irregular dispersion and clustering. So the test is to estimate distribution in space or on a plane and then to perform suitable statistical tests to see whether the distribution agrees with or deviates from a Poisson distribution.