MORPHOMETRIC STUDY OF TROUT GILLS:
A LIGHT-MICROSCOPIC METHOD SUITABLE FOR THE EVALUATION OF POLLUTANT ACTION

BY G. M. HUGHES AND S. F. PERRY*

Research Unit for Comparative Animal Respiration, Bristol University

(Received 27 August 1975)

SUMMARY

1. Methods are described for the morphometric estimation of parameters of the gill system of trout which are relevant to its function in gas exchange. The methods have been used with 1 μm sections viewed under the light microscope.

2. In particular the diffusion distances between water and blood are measured, which together with determinations of gill area, provide figures for the morphometrically estimated diffusing capacity.

3. The methods have been used to compare the diffusing capacity of gills from control fish and those treated in polluted waters. The concept of relative diffusing capacity ($D_{rel}$) is introduced which enables comparisons to be made without the need to determine the absolute diffusing capacity.

4. Quantitative estimation of changes in relative volumes and surface areas of components of the secondary lamellae were determined, and employed to explain the possible anatomical causes of changes in $D_{rel}$.

5. It is suggested that these methods can be of value in the comparison of the gills of fish treated in different waters.

INTRODUCTION

During recent years much detailed information has been obtained regarding the morphology of fish gills (Hughes & Grimstone, 1965; Hughes & Wright, 1970; Morgan, 1974; Morgan & Tovell, 1973; Tovell, Morgan & Hughes, 1970; Wright, 1973). These studies, although mainly morphological, have also drawn attention to some of the dimensions of the water/blood barrier, which separates the blood from the water in which the fish is living. In many cases these barriers are extremely thin (e.g. less than 1 μm in some tuna gills). There have also been studies which indicate the ways in which the basic gill morphology is influenced by pollutants contained in the water breathed by the fish (Herbert & Merkens, 1961; Brown, Mitrovic & Stark, 1968; Marchetti, 1969; Camatini & Lanzavecchia, 1970; Skidmore & Tovell, 1972). Throughout these studies the need for quantitative methods for making these measurements has become apparent and more recently a number of studies have been in this direction.

* Present address: Zentrum für Anatomie und Cytobiologie, Justus Liebig Universität, Giessen, Germany.
Morphometric methods for mammalian lungs based upon measurements from electron micrographs of randomly oriented sections have been developed (Weibel, 1971). Some possible methods and their application using electron microscopy to gills of the tench have already been discussed (Hughes, 1972). One disadvantage of electron microscopic studies is that they are based on a relatively small sample from the whole gill system. Consequently methods based on light microscopy, if they could be carried out with sufficient accuracy, would be more ideal for gaining information which was valid for the greater part of the gill system. This paper presents methods in which this possibility has been explored and uses methods which take account of the highly organized nature of the gill system and the difficulty of making a randomized selection of material.

MATERIALS AND METHODS

Rainbow trout (Salmo gairdneri) had been reared at the Water Research Laboratory, Stevenage, and kept in pollutants of known strength. Gills were fixed either at Stevenage or following transport of fish to Bristol and maintenance in aquaria for varying recovery periods. Control fish, kept under identical conditions but with no pollutants, were also available for each treatment. The weight range of the fish was from 13 to 53 g with a mean of 35 g.

The fish were stunned by a sharp blow on the head and the brain severed behind the eyes by means of bone cutters. The fish was held in a vertical position and glutaraldehyde fixative poured through the mouth and over the gills for about 1 min. The fixative was 0.25 M glutaraldehyde in 0.04 M cacodylate buffer at pH 7.5. The osmolarity was adjusted with sucrose to 350 m-osmole. Samples of filaments from the central part of the second and third arches were removed and placed in the fixative. In two control fish, samples from all the gill arches were taken in order to investigate any differences in control samples. The tissue was stored in fixative overnight at 4 °C, post-fixed for 1 h in 1% OsO₄ in cacodylate buffer, dehydrated, and prepared for embedding in epon the following day.

One-μm sections were cut using an LKB ultratome. Routinely two or three blocks, each containing one filament, were sectioned for each fish. Detailed morphometric analysis was carried out on a single section from each of these blocks.

Inspection through the dissecting microscope ensured that the sections were, as far as possible, perpendicular to the surface of the secondary lamellae. Additional control of sectioning artifacts was obtained by counting the frequency of secondary lamellae in the block and once more in the sections. If the percentage difference between these two counts exceeded 10%, these sections were not used. Such differences could be due to differences in the angle of the sections, or to compression of the material during sectioning. The sections were stained with toluidine blue and mounted in DPX. Slides were viewed under a Wild automatic sampling microscope using oil immersion at a magnification of 1413 ×.

Model

Figs 1 and 2 indicate subdivisions of the gill system which were adopted in the present study. Sections of the gill filaments were divided into the lamellar
Fig. 1. Longitudinal section through the apical portion of a gill filament, showing division into the lamellar region, LR, and the filamentar axis, F. LR includes both the secondary lamellae and the interlamellar spaces. OLR indicates the space outside the lamellar region and was not of interest in this work.
region and filamentar axis. The former consists of the sections of the secondary lamellae and water in the interlamellar spaces. Within the secondary lamellae themselves a distinction was made between the pillar cell system (PS) and the portions outside the basement membranes. The region outside the pillar cell system (OPS) is divisible into a cellular compartment usually composed of two epithelial layers, and the non-cellular space which is frequently found between them. The PS is formed of the basement membranes together with the pillar cells and their flanges, which are within the basement membranes. It also includes the marginal channels, which are partly lined by endothelial cells. The blood filling the channels between pillar cells and the marginal channel consists of large nucleated red cells, white cells, and blood plasma. Under the light microscope all of these components were distinguishable at the magnification used \((1413 \times)\), and point and intersection counting could readily be made. This model is appropriate not only to the normal gill but also in relation to modifications in gill structure that result from the particular treatments investigated in the second part of this study (Hughes, Perry & Brown, 1975).

### Table 1. Abbreviations and definition of terms

<table>
<thead>
<tr>
<th>Position</th>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Anatomical regions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within LR</td>
<td>F</td>
<td>Axis of filament</td>
</tr>
<tr>
<td></td>
<td>OLR</td>
<td>Water space outside lamellar region</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>Lamellar region</td>
</tr>
<tr>
<td>Within SL</td>
<td>OSL</td>
<td>Water space between secondary lamellae</td>
</tr>
<tr>
<td></td>
<td>SL</td>
<td>Secondary lamellae</td>
</tr>
<tr>
<td>Within OPS</td>
<td>nt</td>
<td>Non-tissue spaces</td>
</tr>
<tr>
<td></td>
<td>tis</td>
<td>Tissue-occupied regions</td>
</tr>
<tr>
<td>Within PS</td>
<td>bm</td>
<td>Basement membrane</td>
</tr>
<tr>
<td></td>
<td>pc</td>
<td>Pillar cell, including body and flanges</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>Blood</td>
</tr>
<tr>
<td>Within b</td>
<td>e</td>
<td>Erythrocytes</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>Plasma, white blood cells</td>
</tr>
<tr>
<td>Structures containing blood</td>
<td>bc</td>
<td>Blood channels, bounded on all sides by pillar cells</td>
</tr>
<tr>
<td></td>
<td>mc</td>
<td>Marginal channels</td>
</tr>
<tr>
<td>2. Measured distances</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>Total distance from a point on the secondary lamellar surface to the nearest erythrocyte</td>
<td></td>
</tr>
<tr>
<td>l'</td>
<td>That portion of l composed of cells and connective tissue</td>
<td></td>
</tr>
<tr>
<td>l_{nt}</td>
<td>That portion of l composed of non-tissue space</td>
<td></td>
</tr>
<tr>
<td>τ</td>
<td>Harmonic mean of l values</td>
<td></td>
</tr>
<tr>
<td>τ'</td>
<td>Harmonic mean of l' values</td>
<td></td>
</tr>
</tbody>
</table>

### Morphometric methods

The purpose of these methods was (1) To evaluate the diffusing capacity of the membranes morphometrically in order that this parameter may be compared for experimental and control fish; (2) to compare the relative volumes of components within the secondary lamellar region of control and experimental fish; (3) to compare
relative surface ratios within the secondary lamellar region of control and experimental fish.

The number of intersections per unit area of a non-oriented test line system with cut surfaces in a section is directly proportional to the surface area of the same structure per unit volume (Underwood, 1970). In the case of a lung, where the alveolar surfaces are randomly oriented, a system of oriented test lines can be used (Weibel, 1971). In the present case, however, where the sections were cut in a regular relationship to the secondary lamellar surfaces, a system of non-oriented, semi-circular test lines (Merz, 1967) was used. Similarly, if a non-oriented system of points is superimposed on a section of negligible thickness, the number of points falling on the profile of a certain structure per unit reference area is directly proportional to the volume of those structures per unit reference volume. Such a system of points was also present on the test line system used (Fig. 3) and thus enabled the experimenter to obtain intersection counts and point counts from the same field and for exactly the same reference area. All measurements were made using 1 μm Epon sections and a Wild projection microscope at a magnification of 1413 x. This system provided sufficiently good resolution for measurement of distances from intersections of the test lines with the secondary lamellar surface to the nearest erythrocyte.

Values of morphometric diffusing capacity ($D_{\text{morph}}$) and the concept $D_{\text{rel}}$

Diffusing capacity is a physiological parameter defined as the quantity of a substance, e.g. oxygen, which passes across a membrane system in unit time for a given partial pressure difference. In the case of a water-breathing fish, $D_{O_2} = \frac{V_{O_2}}{\Delta P_g}$, where $\Delta P_g$ is the mean difference in $P_{O_2}$ between the water and blood in the gills. Morpho-
Fig. 3. Portion of the lamellar region, with Merz grid superimposed. The size of the grid and magnification of the photomicrograph about half those used. Some measurements, points and intersections are indicated. $l = \text{total water-blood distance}$, $l_{nt} = \text{portion of } l \text{ in OPS not occupied by tissue}$, $l_{o} = \text{intersection with outer surface of secondary lamella}$, $l_{i} = \text{intersection with 'inner' surface formed by the basement membrane}$, $P_{bm} = \text{point lying over tissue component of OPS}$, $P_{nt} = \text{point lying over non-tissue component of OPS}$, $P_{bm \& nt} = \text{point lying over either basement membrane or pillar cell component of PS}$. Epon embedded: $1 \mu m$ section, stained with toluidine blue.
Morphometry of trout secondary lamellae

metrically, this is equivalent to the relationship $D = (KA)/t$, where $K$ is the Krogh permeation coefficient, being the product of the diffusion coefficient ($D$) and the solubility ($a$). Unfortunately, there is relatively little data on these coefficients in living systems and most of the values used originate from the early work of Krogh (1919). Clearly, it is probable that different coefficients apply to different portions of the overall barrier to gas transfer, but as yet there is little data to indicate the magnitude of such differences. For the barrier to gas transfer the overall diffusing capacity has been subdivided into components as follows (Hughes, 1972):

$$\frac{1}{D_g} = \frac{1}{D_w} + \frac{1}{D_m} + \frac{1}{D_l} + \frac{1}{D_p} + \frac{1}{D_o}.$$  

In the present investigation a simplified approach has been adopted in which the overall resistance to gas transfer between the outer secondary lamellar surfaces and the red blood cell membrane is regarded as a single unit, equivalent to $D_l + D_p$ and designated here $D_{lam}$. In addition $D'_{lam}$, from which the so-called lymphoid spaces were excluded, was also calculated for comparison.

One of the main interests of the present work is in relative diffusing capacity ($D_{rel} = D_{exp}/D_{con}$) as a function of the effective pollutant concentration. This has been expressed as

$$\frac{K_{exp} \times \text{area/thickness of experimental gills}}{K_{con} \times \text{area/thickness of control gills}}.$$  

Assuming that the permeation coefficient ($K$) for both polluted and control fish remains the same then these constants can be ignored. However, if there are differences in the constants for different parts of the system this would clearly not be a valid assumption. In the data plotted, $D_{rel}$ including the lymphoid space and $D'_{rel}$ in which the contribution of the lymphoid space has been omitted, have been plotted separately to see if any trends were apparent, assuming that the tissue portion of control and polluted fish remains the same. Thus, with these assumptions in mind, it is apparent that $D'_{rel}$ can act as a sort of control against which the changes in $D_{rel}$ can be compared. For the morphometric calculation of absolute values of the overall diffusing capacity it is essential to know both $K$ and the total gill surface area, as well as certain properties of the blood, especially the reaction velocity ($\theta$) between oxygen and haemoglobin in the red blood cells.

**Formulae for calculation of absolute $D_{Ox}$ ($D_{morph}$)**

$$\frac{1}{D_{morph}} = \frac{1}{D_{lam}} + \frac{1}{D_e}, \quad (1)$$

$$D_{lam} = K_{lam} \frac{A I_{O \exp}/I_{O \con}}{\tau_{lam}}, \quad (2)$$

where $K_{lam}$ is a single permeation coefficient for the whole barrier which has a harmonic mean thickness of $\tau_{lam}$ and a gill area defined from the allometric relationship $A = aW^b$ modified according to the ratio of intersection counts for the outer surfaces of the secondary lamellae in the experimental and control fish ($I_{O \exp}/I_{O \con}$). Values for total secondary lamellar area of control fish were estimated from the
slope of the regression line relating gill area \( A \) and body weight for other specimens (Hughes, unpublished). An appropriate \( K \) value for oxygen in gill tissue is approximately \( 2.0-2.4 \times 10^{-8} \text{ cm}^2/\text{min/mmHg at 15 }^\circ\text{C} (= 2.0-2.4 \times 10^{-4} \text{ ml/cm}^2/\mu\text{m/min/mmHg}). \)

Regarding the diffusing capacity of the erythrocytes,

\[
D_0 = \theta V_{e}/Ht_{Hb},
\]

\[
V_e = \left( \frac{V_{e}}{V_{SL,exp}} \right) \left( \frac{V_{SL,exp}}{S_{O,exp}} \right) \left( \frac{S_{O,con}}{S_{O,con}} \right) A_0, \quad (3a)
\]

\[
Ht_{Hb} = 0.88V_e/V_b. \quad (3b)
\]

In equation \((3a)\) the expressions in parentheses were determined as ratios of intersections or point counts, and \( A_0 \) is equivalent to the total secondary lamellar surface area \( (S_{O,con}) \) of a trout of the appropriate weight as determined from the allometric equation. Equation \((3a)\) can be rewritten as

\[
V_e = V_{e} A_0 \frac{I_{0,exp}}{I_{0,con}} S_{SL}^{-1},
\]

\( S_{SL} \) is the secondary lamellar surface density with respect to the secondary lamellar volume for the specimen in question. \( V_{e} \) is the volume density of the erythrocyte with respect to secondary lamellar volume. For calculation of \( Ht_{Hb} \) it has been assumed that 88% of the erythrocyte volume is occupied by haemoglobin, as has been found for \textit{Testudo} blood (Perry, unpublished). \textit{Haematocrit} (\( Ht \)) for the secondary
Morphometry of trout secondary lamellae

453

•lamellae was estimated morphometrically as $P_e/P_e + P_p$. Values for $\theta$ in nucleated erythrocytes have not yet been determined. However, preliminary information (Hughes & Koyama, 1975) indicates that is a reasonable estimate.

Relative volumes and surface areas of components of the secondary lamellae

Relative diffusing capacity ($D_{rel}$) and harmonic mean thickness indicate overall changes which, from an anatomical point of view, affect the function of secondary lamellae in gas exchange. However, knowing relative volumes and surfaces of the various secondary lamellar constituents, the nature of changes following the experimental treatment can be described more precisely. Point counting makes it possible to estimate volumes of the different components of the tissue within a reference volume (i.e. volume densities). Points that were counted in the present study are shown in Fig. 4. By adding together counts for individual parameters, volume densities concerning larger units within the system may be derived, e.g.

$$\frac{V_b}{V_{SL}} = \frac{P_e + P_p}{P_e + P_p + P_{bm+pc} + P_{dias} + P_{nt}}.$$

The volume densities which are of particular interest include the following parts of the pillar cell system, which are conveniently expressed relative to (a) the total secondary lamellar volume:

$$V_{bm+pc}/V_{SL}, \quad V_e/V_{SL}, \quad V_b/V_{SL},$$

or (b) the volume of the pillar cell system:

$$V_{pm+pc}/V_{PS}, \quad V_e/V_{PS}, \quad V_b/V_{PS}.$$

The third group of volume densities studied were as follows:

$$\frac{V_{SL}}{V_{LR}} = \text{proportion of lamellar region occupied by secondary lamellae.}$$

$$\frac{V_{OPS}}{V_{SL}} = \text{proportion of a lamella outside the pillar cell system.}$$

$$\frac{V_{dias}}{V_{OPS}} = \text{proportion of volume outside the pillar cell system that is composed of tissues.}$$

Secondary lamellar haematocrit is expressed as $Ht = P_e/P_{blood}$ or $0.88 P_e/P_{blood}$ for $Ht_{HB}$.

Ratios between the surfaces of different parts of the system can be measured by means of the intersections of their surfaces with the lines of the Merz grid (Fig. 4). Surfaces estimated were as follows:

(i) outer surface of the secondary lamellae ($S_0$);
(ii) basement membrane, i.e. internal surface, overlying pillar cells ($S_{1pc}$);
(iii) overlying blood space ($S_{1b}$) and
(iv) overlying the marginal channels ($S_{1mc}$).

By expressing these as ratios of one surface to another, comparisons can be made between different gill specimens, as systematic errors due to differences in the plane of the sections are minimized. The ratios of interest in investigations of this kind are as follows:

1. $S_t/S_0$ If $S_t/S_0$ of controls $= 1$, then $S_t$ for a ‘polluted’ fish can serve as an internal control, i.e. to indicate what $S_0$ might have been had the fish not been treated.
G. M. HUGHES AND S. F. PERRY

(2) \( S_{t_{bc}}/S_I = \) proportion of \( S_I \) over blood channels.

(3) \( S_{t_{mc}}/S_I = \) proportion of surface made up by marginal channels had the fish not been treated.

(4) \( S_{t_{mc}}/S_0 = \) proportion of the actual surface made up by the marginal channel (\( S_{t_{mc}}/S_0 \) is theoretically better but, \( S_{t_{mc}} \) was not measured in the present study).

RESULTS

Calculation of \( D_{O_2} \) and comparison with previous data

\( D_{lam} \)

The derived secondary lamellar surface area (\( A \)) for a rainbow trout of 35 g is 91 cm\(^2\) or 2.6 cm\(^2\)/g. \( J_O/I_I \) (exp) was used\(^*\) to represent \( J_O/exp/I_O/con \) and \( K_{O_2} \) was assumed to be \( 2.0 \times 10^{-8} \) or \( 2.4 \times 10^{-8} \) cm\(^3\)/min/mmHg. Substituting these values and the appropriate values for \( \tau_{lam} \) into equation (2), \( D_{lam} \) was obtained as shown in Table 2.

\( D_{rel} \) and analysis of volume and surface ratios

\( D_{rel} \) indicates the diffusing capacity (\( D_{lam} \)) of one group of animals relative to that of a control group for a standard volume of the secondary lamellar region (LR), but gives no information about the reason for the differences. If \( D'_{rel} \) is greater than \( D_{rel} \), one suspects a greater non-tissue component in OPS for the experimental group than for the control. In order to define the cause of the reduction in \( D_{rel} \), \( \tau \) and the relative volumes and surfaces were examined. In the present example, exposure to nickel appears to have increased the water–blood distance (\( \tau \)) and decreased the secondary lamellar surface area (\( S_I/S_0 = 1.82 \) and 4.77 following treatment with 2.0 and 3.2 mg/l nickel and 1.02 for controls). That the surface area was decreased by fusion of the secondary lamellae is indicated by the large increase in \( V_{SL}/V_{LR} \) (0.71, 0.94; 0.60 in controls). Inspection of haematocrit and the relative volumes of the structures within the pillar cell system (PS) eliminates them as possible sources of the increase in \( \tau \). However, the volumes of the same structures relative to that of the whole secondary lamellae (SL) is lower than in the controls, especially in the case of the higher dosage of nickel. This decrease can be caused by contraction of the pillar cells or by an absolute increase in the volume of OPS. \( V_{PS}/V_{SL} \) is obtained as \( 1 - V_{OPS}/V_{SL} \). By multiplying \( V_{PS}/V_{SL} \) by \( V_{SL}/V_{LR} \) one obtains \( V_{OPS}/V_{LR} \), by which the relative size of PS can be detected independently of the swelling of OPS. The values obtained (0.280, 0.256, 0.193 for 0, 2.0 and 3.2 mg/l nickel, respectively) indicate a reduction in the size of PS at the higher nickel dosage.

Volumes outside the pillar cell system also showed changes. Thus \( V_{OPS}/V_{SL} \) increases after nickel treatment, as does \( V_{OPS}/V_{LR} \), calculated as \( V_{OPS}/V_{SL} \times V_{SL}/V_{LR} \) (0.320, 0.455, 0.030 = \( S_I/S_0 \), (see \( S_I/S_0 \), Table 3) (1)

\[ I_{exp} = I_{con} \] (indicated because \( V_{con+exp}/V_{PS} \), \( S_{t_{bc}}/S_I \) (2)

and \( S_{t_{mc}}/S_I \) for experimental animals are similar to control values, i.e. the pillar cell system anatomy is not affected by the nickel treatment).

Combining (1) and (2): \( I_{exp} = I_{con} \). Substituting into the expression \( I_{exp}/I_{con} \):

\[ I_{exp}/I_{con} = I_0/exp/I_0/con \] or \( I_0/\tau \) (exp). (3)
Morphometry of trout secondary lamellae

for o, 2.0 and 3.2 mg/l nickel, respectively). This enlarged OPS appears to have greater non-tissue component than does the OPS of the control, as shown by V_{mus}/V_{OPS}. Nevertheless, when expressed as V_{mus}/V_{LR} calculated as V_{mus}/V_{OPS} \times V_{OPS}/V_{SL} \times V_{SL}/V_{LR}, an increase in the amount of tissue in the OPS region after nickel treatment is demonstrated (0.261, 0.335, 0.535 for o, 2.0 and 3.2 mg/l nickel, respectively).

Regarding the ratios of surfaces, the suitability of S_I as an indicator of the expected S_O is shown by S_I/S_O = 1.0 in the control group. That the integrity of the pillar cell system was not disturbed by exposure to nickel is shown by the relatively constant proportion of S_I which is located over the blood channels (S_{bc}/S_I). The potential contribution of the marginal channels is shown to increase from approximately 6 to 8% of S_I to 42% of the respiratory surface area (S_O) after exposure to 3.2 mg/l nickel.

DISCUSSION

One of the main results of this work is the demonstration that morphometry based only on light microscopy can be applied to the analysis of the secondary lamellar system of trout. Such methods have the advantage of sampling a larger proportion of the total gill surface, whereas studies based on electron microscopy inevitably use far smaller portions of the total gill sieve. Electron microscopy is preferable where small dimensions are to be measured, e.g. the harmonic mean barrier thickness. But for the estimation of surface and volume densities of such regions as inside and outside the pillar cell systems, light microscopy is to be preferred.

The higher values for D_{lam} of the controls in the present study as compared with D_I of Hughes (1972) are attributed to the use of higher K_{O_2} values (2.0 and 2.4 x 10^{-8} as opposed to 1.5 x 10^{-8} cm^{2}/min/mmHg) as well as to the thinner water-blood distance (4.3 as opposed to 6.0 μm). In addition, A in the present study is calculated for fish of 36 g, which have a higher A per unit body weight than the 1000 g animals used in the previous calculations (2.6 as opposed to 2.0 cm^{2}/g).

In this study only two samples have been used from each of two fish for each treatment and the standard deviations were approximately 20–30% of the mean values. With such small numbers the technique is accurate enough to show trends, but probably not sufficiently accurate to allow reliable statistical analysis. If the number of fish per group were increased to four or five the sensitivity would probably increase considerably.

In the calculations made here, the following assumptions have been made with respect to the measurements of oxygen diffusion:

(a) oxygen molecules only enter through the secondary lamellar surface,
(b) oxygen molecules have an equal probability of entering at any point on the secondary lamellar surface,
(c) the path of diffusion is the shortest distance from the point of entry to the surface of the nearest erythrocyte, and
(d) there is no facilitation of gas transport by convection within the plasma.

With respect to (c) and (d) it is clear that convection in the plasma may greatly accelerate the transfer of oxygen to the red blood cell and therefore the effective K
for this part of the pathway may be greater than that anticipated simply by a consideration of direct gas diffusion. No allowance is made for convection in the present analysis, and any attempt to make a quantitative estimate of its effect would be complicated by the fact that it will vary in different channels of the secondary lamellae (Hughes, 1972, 1975) because of their heterogeneity.

During the present investigation measurements of barrier thickness were made between the outer surface of the secondary lamellae and the nearest red blood cell but no estimate was made of different parts of this pathway. Thus, the figures summarized here referred to as $D_{\text{lam}}$ differ from those for both $D_t$ and $D_g$ that have been estimated in previous studies (Hughes, 1972). One disadvantage of the present method is that it assumes that the blood is fixed in a condition which faithfully reproduces the dynamic state of circulation in the intact gills. Clearly it is doubtful that such a situation could be achieved by the method of fixation adopted here. An estimate for the proportion of the harmonic distance attributable to the pathway between the wall of the blood channels and the red blood cells is about 0.50 μm. If allowance is made in the calculations for this reduction in $\tau$, the figures can be more related to those of $D_t$.

It is a common feature of morphometric investigations of this kind that a particular
**Morphometry of trout secondary lamellae**

Table 2. Calculation of morphometric diffusing capacity for control and experimental rainbow trout

<table>
<thead>
<tr>
<th>Dosage (mg nickel/l)</th>
<th>$A/I_1$ ($\text{cm}^2/\text{g}$)</th>
<th>$\tau_{bn}$ ($\mu$m)</th>
<th>$D_{bn, o_b}$ ($\text{cm}^3/\text{min. kg. mmHg}$)</th>
<th>$D_{l, o_b}$ ($\text{cm}^3/\text{min. kg. mmHg}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.55</td>
<td>4.31</td>
<td>0.118-0.142</td>
<td>0.05</td>
</tr>
<tr>
<td>2.0 mg/l nickel</td>
<td>1.43</td>
<td>7.63</td>
<td>0.037-0.045</td>
<td></td>
</tr>
<tr>
<td>3.2 mg/l nickel</td>
<td>0.543</td>
<td>9.15</td>
<td>0.012-0.014</td>
<td></td>
</tr>
</tbody>
</table>

Hughes, 1972

---

Table 3. Summary of morphometric data on trout secondary lamellae

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Dosage (mg nickel/l)</th>
<th>0</th>
<th>2.0</th>
<th>3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{re}$</td>
<td>Diffusing capacity relative to that of control animals</td>
<td>—</td>
<td>0.145</td>
<td>0.124</td>
<td></td>
</tr>
<tr>
<td>$D'_{re}$</td>
<td>Diffusing capacity relative to that of control animals, where non-tissue spaces have been neglected</td>
<td>—</td>
<td>0.405</td>
<td>0.142</td>
<td></td>
</tr>
<tr>
<td>$\tau$ ($\mu$m)</td>
<td>Harmonic mean distance from the secondary lamellar surface to the nearest erythrocyte</td>
<td>4.313</td>
<td>7.633</td>
<td>9.153</td>
<td></td>
</tr>
<tr>
<td>$\tau'$($\mu$m)</td>
<td>Harmonic mean distance from the secondary lamellar surface to the nearest erythrocyte, neglecting non-tissue spaces</td>
<td>3.563</td>
<td>5.635</td>
<td>7.313</td>
<td></td>
</tr>
<tr>
<td>$HT$</td>
<td>Secondary lamellar hematocrit</td>
<td>0.537</td>
<td>0.479</td>
<td>0.588</td>
<td></td>
</tr>
<tr>
<td>$V_{lam+ps}/V_{sl}$</td>
<td>Proportion of the secondary lamellar volume occupied by basement membrane and pillar cells</td>
<td>0.141</td>
<td>0.137</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>$V_d/V_{sl}$</td>
<td>Proportion of the secondary lamellar volume occupied by erythrocytes</td>
<td>0.168</td>
<td>0.110</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td>$V_b/V_{sl}$</td>
<td>Proportion of the secondary lamellar volume occupied by blood</td>
<td>0.327</td>
<td>0.223</td>
<td>0.145</td>
<td></td>
</tr>
<tr>
<td>$V_{lam+ps}/V_{ps}$</td>
<td>Proportion of the volume of the pillar cell system occupied by basement membrane and pillar cells</td>
<td>0.307</td>
<td>0.361</td>
<td>0.296</td>
<td></td>
</tr>
<tr>
<td>$V_d/V_{ps}$</td>
<td>Proportion of the volume of the pillar cell system occupied by erythrocytes</td>
<td>0.362</td>
<td>0.304</td>
<td>0.418</td>
<td></td>
</tr>
<tr>
<td>$V_b/V_{ps}$</td>
<td>Proportion of the volume of the pillar cell system occupied by blood</td>
<td>0.693</td>
<td>0.639</td>
<td>0.704</td>
<td></td>
</tr>
<tr>
<td>$V_{sl}/V_{lb}$</td>
<td>Proportion of the volume of the secondary lamellae</td>
<td>0.600</td>
<td>0.711</td>
<td>0.942</td>
<td></td>
</tr>
<tr>
<td>$V_{ps}/V_{sl}$</td>
<td>Proportion of the volume of the secondary lamellae occupied by non-tissue spaces and by tissue exterior to the basement membranes</td>
<td>0.533</td>
<td>0.640</td>
<td>0.795</td>
<td></td>
</tr>
<tr>
<td>$V_{lw}/V_{ps}$</td>
<td>Proportion of the region exterior to the basement membranes of the secondary lamellae which is occupied by tissue – i.e. non-tissue spaces excluded</td>
<td>0.817</td>
<td>0.736</td>
<td>0.715</td>
<td></td>
</tr>
<tr>
<td>$S_l/S_0$</td>
<td>Surface area of the basement membrane relative to the surface area of the secondary lamellar epithelium</td>
<td>1.018</td>
<td>1.821</td>
<td>4.768</td>
<td></td>
</tr>
<tr>
<td>$S_{lam}/S_l$</td>
<td>Surface area of the blood channels relative to the surface area of the basement membrane</td>
<td>0.746</td>
<td>0.798</td>
<td>0.740</td>
<td></td>
</tr>
<tr>
<td>$S_{lam}/S_l$</td>
<td>Surface area of the marginal channels relative to the surface area of the basement membrane</td>
<td>0.079</td>
<td>0.061</td>
<td>0.076</td>
<td></td>
</tr>
<tr>
<td>$S_{lam}/S_0$</td>
<td>Surface area of the marginal channels relative to the surface area of the secondary lamellar epithelium</td>
<td>0.080</td>
<td>0.120</td>
<td>0.416</td>
<td></td>
</tr>
</tbody>
</table>
model is used upon which the measurements are based. That used in the present work is shown in Fig. 2, but the final result is obtained in terms of mean values for different portions of the system and may be summarized in the model illustrated in Fig. 5. It is apparent that this gives a picture of uniform thickness for each layer which is clearly an abstraction from the real situation. Thus, for trout the intra-epithelial spaces are never uniformly present along the whole length of the secondary lamellae, as indicated in this diagram. Perhaps it is even more important, however, to be aware that the picture given by these figures is based upon such a model rather than that with which the study commenced.

The model used here is essentially the same as that taken by Weibel (1971) for the mammalian lung with the exception that a single permeation coefficient is used for both tissue and plasma. A value of \(2 \times 10^{-8} \text{ cm}^2/\text{min/mmHg} \) at 15 °C was taken as an average of values for plasma (Grote, 1967) and frog connective tissue (Krogh, 1919) slightly reduced because of the 10 °C difference from the temperatures of the original determinations.

It will be noted that the method used for calculating \(D_{rel} \) assumes that the arrangement of the secondary lamellae within the lamellar region is similar in all specimens. If during the fixation procedure the secondary lamellae of some specimens became collapsed on each other, this would lead to final values for \(D_{rel} \) which were too high. In most of the specimens used in this work fixation did not result in such a collapsed condition of the secondary lamellae and consequently few errors of this kind were encountered.

However, if the danger of secondary lamellar collapse is greater, then an alternative method for calculating \(D_{rel} \) should be adopted which is independent of such changes. Such a method might involve the use of \(I_T \) as a reference instead of LR used in the present system. Thus instead of calculating \(D_{rel} \) as \(\left(\frac{S_{exp}/T_{exp}}{S_{con}/T_{con}} \right) \), it may be expressed as

\[
\frac{(S_0/S_1)_{exp}}{T_{exp}} \frac{(S_0/S_1)_{con}}{T_{con}}.
\]

The results of these two methods for estimating \(D_{rel} \) using values from Table 3, as well as one example from Hughes, Perry & Brown (1975) are shown below:

<table>
<thead>
<tr>
<th>Method</th>
<th>2.0 mg Ni/l</th>
<th>3.2 mg Ni/l</th>
<th>19 days recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard = LR</td>
<td>0.41</td>
<td>0.12</td>
<td>1.50</td>
</tr>
<tr>
<td>Standard = (S_1)</td>
<td>0.32</td>
<td>0.10</td>
<td>1.03</td>
</tr>
</tbody>
</table>

The discrepancies between the results obtained by these two methods can be large, but should decrease with an increase in sample size. In general, the use of a standard LR is recommended if it is uncertain whether the pillar cell system has been damaged by the pollutant treatment. Cases in which the histological procedures have caused collapse of the secondary lamellae should be discarded.

The values obtained for surface densities and ratios are computed for structures within the same filament. It should therefore be independent of errors caused by differences in the plane of sectioning, provided that no sections pass parallel to the
secondary lamellar surface. There is little chance of this occurring since care was taken to cut sections as nearly as possible perpendicular to the surface, and error factors were computed.

The methods described in the present paper were designed both to obtain quantitative information about the normal gill structure and also in order to quantify the effects of foreign substances on the gills. They have shown the ways in which pollutants such as nickel can affect the diffusing capacity, and how changes in diffusing capacity can be explained in terms of quantitative changes in relative volumes and surfaces of the system. These results are possible using sectioned material alone. In order to obtain absolute values for diffusing capacity, etc., it is essential to know the total gill area. As mentioned earlier, the methods described here are probably less accurate than those based on electron microscopy but they are faster and better suited for comparative studies than for the more detailed studies of normal gills.

To summarize the advantages of this technique:

1. it is more rapid than electron microscopy;
2. the Epon-embedded tissue can be thin sectioned if it is required for future electron microscopy;
3. one-micron sections are thin enough for the Holmes effect to be relatively unimportant and shrinkage is probably also unimportant. Shrinkage in some paraffin-sectioned material of dogfish secondary lamellae may be as great as 10% in area (Hughes, Perry & Piiper, unpublished observations).

The main obstacles are that it requires a high-quality projection microscope and that, like all morphometric techniques, is time-consuming. With these limitations in mind it seems possible that the methods described in this paper can fulfil a useful purpose in quantifying the effects of environmental factors, including pollutants, on the secondary lamellae of fish gills. It is clearly important to gain information as to which of the many parameters measured in this study are most affected by a particular environmental change. In this way the number of structures which are to be measured and counted can be limited and a larger sample of material can be processed, thus increasing the sensitivity of the method.

This work was supported by a grant from the Natural Environment Research Council which is gratefully acknowledged. We thank Robert Sully for his expert photographic assistance.

We wish to thank Mr J. Alabaster and other members of his staff at the Stevenage Water Research Centre for their help throughout this work.

REFERENCES


