PHYSICAL STRUCTURE OF SHELL MEMBRANES

BY J. J. WOLKEN AND F. A. SCHWERTZ

(From the Department of Biological Sciences, University of Pittsburgh, and The Mellon Institute of Industrial Research, Pittsburgh)

(Received for publication, May 19, 1948)

INTRODUCTION

For the metabolic processes involved in the development of the chick embryo, an adequate supply of respiratory gases is required. Several experimenters have shown that the air enters initially through small holes in the egg shell. Romanoff (1) studied, in addition, the permeability to air and other gases of the lamination formed from both the shell and shell membrane and found values of the order of several million times those obtained from high polymer membrane such as natural rubber. Moreover, he found that, generally speaking, the lighter gases were more penetrating than the heavy ones. These two facts, taken together, indicated that the gas transfer probably took place through small holes in both the shell and shell membrane. Since the presence of holes in the shell had been carefully demonstrated by Marshall and Cruikshank (2), the present authors thought it would be of interest to study the shell membrane alone. This membrane consists of two distinct layers which are everywhere in contact except at the rounded part of the egg where they separate to form an air sack (3). The results obtained show clearly that dried shell membranes do indeed contain very small holes and that a very rough estimate of their mean size may be obtained by gas permeability measurements alone. This estimate has been verified by electron microscope pictures of the shell membrane.

The Permeability Method

The transfer of gas through a film containing capillary pores may take place by true viscous flow, or by diffusion, or by a combination of these two. In any case, experiment and theory both show that the transfer rate decreases with increasing temperature. On the other hand, if no pores are present in the film, the transfer of gas will take place by interstitial diffusion of the activated type, the rate of which increases exponentially with the temperature. Our method of detecting the presence of pores in the shell membrane consisted simply in taking permeability measurements at two different temperatures for a series of common gases including H₂, He, N₂, O₂, and air. The permeability for all these gases showed a decrease with increasing temperature.
Experimental Arrangement and Procedure

The essential part of the experimental set-up is shown in Fig. 1. This whole permeability unit was placed in a water bath whose temperature was thermostatically controlled. Also in the water bath was placed a 10 foot coil of \( \frac{1}{4} \) inch copper tubing through which the gas was passed before entering the permeability unit. This arrangement insured that the temperature of the gas in contact with the membrane was the same as that of the bath.

The shell membrane was stripped from the shell and mounted between two ground-glass plates, as illustrated in Fig. 1. The subassembly containing the membrane was then placed in a desiccator and dried for at least 24 hours. In the drying process the membrane stuck firmly to the ground-glass plates, thereby forming a gas-tight seal.
The experimental procedure consisted simply of passing gas through the membrane under a known pressure difference and at a known temperature, and of determining the rate of passage of the gas by collecting it over water in a pneumatic trough. A series of gases difficultly soluble in water was first run through the membrane at room temperature and at fixed pressures. The temperature was then increased about 15° or 20°C., but the pressures were left unchanged and the procedure was repeated. To check the reproducibility of the data, some of the experiments were repeated. The membrane was checked for macroscopic fissures and cracks at the end of the experiments by saturating it with water. In this condition, an undamaged membrane was completely impervious to all gases.

**Summary of the Findings**

Different fresh embryonated hen egg shell membranes were subjected to permeability tests. In each case a circular area of 0.57 cm.² was exposed to

### TABLE I

**Permeability Data on Membrane 1**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Gas used</th>
<th>Gas rate</th>
<th>Temperature</th>
<th>Pressure drop (cm. Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O₂</td>
<td>15.6</td>
<td>23</td>
<td>2.70</td>
</tr>
<tr>
<td>5</td>
<td>O₂</td>
<td>15.2</td>
<td>23</td>
<td>2.70</td>
</tr>
<tr>
<td>2</td>
<td>N₂</td>
<td>18.6</td>
<td>23</td>
<td>2.70</td>
</tr>
<tr>
<td>6</td>
<td>H₂</td>
<td>42.6</td>
<td>23</td>
<td>2.70</td>
</tr>
<tr>
<td>4</td>
<td>O₂</td>
<td>14.5</td>
<td>38</td>
<td>2.70</td>
</tr>
<tr>
<td>9</td>
<td>O₂</td>
<td>14.6</td>
<td>38</td>
<td>2.70</td>
</tr>
<tr>
<td>3</td>
<td>N₂</td>
<td>17.6</td>
<td>38</td>
<td>2.70</td>
</tr>
<tr>
<td>8</td>
<td>N₂</td>
<td>17.5</td>
<td>38</td>
<td>2.70</td>
</tr>
<tr>
<td>7</td>
<td>H₂</td>
<td>40.0</td>
<td>38</td>
<td>2.70</td>
</tr>
</tbody>
</table>

The thickness of the membranes varied slightly but all were close to 0.007 cm. The data are summarized in Tables I to III. The number designating the gas rate is in each case the average of from 5 to 10 separate determinations, the gas volume being measured to room temperature and pressure.

**Interpretation of the Data**

The data clearly show that the permeability of the shell membrane to gases decreases with increasing temperature. To draw the conclusion from this fact that the membranes contain micropores requires a closer look into the theory of the transfer of gas through small capillaries. This problem has been carefully studied by Sherwood (4) and his coworkers. They have demonstrated
TABLE II
Permeability Data on Membrane 2
Membrane area = 0.57 cm$^2$, membrane thickness = 0.0065 cm., barometric pressure = 74.5 cm. Hg, room temperature = 23.0°C.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Gas used</th>
<th>Gas rate</th>
<th>Temperature</th>
<th>Pressure drop</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>N$_2$</td>
<td>23.7</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>15</td>
<td>N$_2$</td>
<td>23.8</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>19</td>
<td>N$_2$</td>
<td>24.7</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>11</td>
<td>He</td>
<td>28.6</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>14</td>
<td>He</td>
<td>28.5</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>12</td>
<td>H$_2$</td>
<td>50.2</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>15</td>
<td>H$_2$</td>
<td>49.6</td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>16</td>
<td>N$_2$</td>
<td>21.8</td>
<td>45</td>
<td>3.0</td>
</tr>
<tr>
<td>17</td>
<td>He</td>
<td>26.1</td>
<td>45</td>
<td>3.0</td>
</tr>
<tr>
<td>18</td>
<td>H$_2$</td>
<td>45.4</td>
<td>45</td>
<td>3.0</td>
</tr>
</tbody>
</table>

TABLE III
Permeability Data on Membrane 3
Membrane area = 0.57 cm$^2$, membrane thickness = 0.0065 cm., barometric pressure = 74.7 cm. Hg, room temperature = 22.3°C.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Gas used</th>
<th>Gas rate</th>
<th>Temperature</th>
<th>Pressure drop</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>O$_2$</td>
<td>26.7</td>
<td>24</td>
<td>3.0</td>
</tr>
<tr>
<td>25</td>
<td>O$_2$</td>
<td>28.9</td>
<td>24</td>
<td>3.0</td>
</tr>
<tr>
<td>20</td>
<td>N$_2$</td>
<td>37.9</td>
<td>24</td>
<td>3.0</td>
</tr>
<tr>
<td>24</td>
<td>N$_2$</td>
<td>38.9</td>
<td>24</td>
<td>3.0</td>
</tr>
<tr>
<td>22</td>
<td>H$_2$</td>
<td>89.4</td>
<td>24</td>
<td>3.0</td>
</tr>
<tr>
<td>23</td>
<td>Air</td>
<td>37.3</td>
<td>24</td>
<td>3.0</td>
</tr>
<tr>
<td>27</td>
<td>O$_2$</td>
<td>26.4</td>
<td>37</td>
<td>3.0</td>
</tr>
<tr>
<td>31</td>
<td>O$_2$</td>
<td>25.8</td>
<td>37</td>
<td>3.0</td>
</tr>
<tr>
<td>26</td>
<td>N$_2$</td>
<td>36.6</td>
<td>37</td>
<td>3.0</td>
</tr>
<tr>
<td>30</td>
<td>N$_2$</td>
<td>36.0</td>
<td>37</td>
<td>3.0</td>
</tr>
<tr>
<td>28</td>
<td>H$_2$</td>
<td>84.5</td>
<td>37</td>
<td>3.0</td>
</tr>
<tr>
<td>29</td>
<td>Air</td>
<td>35.3</td>
<td>37</td>
<td>3.0</td>
</tr>
</tbody>
</table>

that the rate of transfer of gases through cylindrical capillaries may be expressed by the single equation

$$n = \frac{\pi D^2 (p_2 - p_1)}{256 \mu LRT} \left[ 1 + 8 \left( \frac{2}{j} - 1 \right) \frac{\lambda_n}{D} \right],$$

where $n$ represents the number of moles of gas passing through the capillary per
In this equation \( \lambda \) is the mean free path of the gas molecules at the mean pressure, \( \rho_m \), in the capillary, \( D \) is the diameter of the capillary, and \( f \) represents the fraction of gas molecules striking the capillary wall that are diffusely reflected. The value of \( f \) is usually in the neighborhood of unity (4). The other symbols have their customary meanings: \( \mu \) is the coefficient of viscosity and is independent of the pressure but not of the temperature, \( L \) is the length of the capillary, \( (\rho_1 - \rho_2) \) the pressure drop across it, and \( T \) is the temperature. For purposes of calculation it is convenient to write the mean free path \( \lambda \) in terms of observable quantities. This may be done with the aid of kinetic theory, which gives

\[
\lambda = \frac{\mu}{\rho_m} \left( \frac{\pi R T}{2 M} \right) .
\]  

(2)

If this value for \( \lambda \) is substituted in equation (1), and if, in addition, equation (1) is separated into two terms, there results

\[
\dot{n} = \frac{\pi D \rho_1 (\rho_2 - \rho_1)}{256 \mu LR T} + \frac{\pi D L (\rho_1 - \rho_2)}{16L \sqrt{2 \pi M R T}} \left( \frac{2}{f} - 1 \right) .
\]  

(3)

Now for a given capillary and fixed values of \( \rho_1, \rho_2, \) and \( T \), the first term on the right-hand side depends only on the viscosity of the flowing gas, whereas the second term is independent of the viscosity and varies inversely as the square root of the molecular weight of the flowing gas. In other words, the first term on the right-hand side of equation (3) represents the transfer of gas by viscous flow, whereas the second term represents the transfer of gas by diffusive flow according to Graham's law of diffusion. The fact which is of singular importance for the present considerations is that both terms in equation (3) decrease with increasing temperature, and, although in any actual membrane we will almost certainly not be dealing with uniform cylindrical capillaries, the pressure and temperature dependence of the flow rate should be given by this equation. We choose, therefore, to write equation (3) in the form

\[
\dot{n} = \frac{a}{\mu T} + \frac{b}{\sqrt{MT}} \left( \frac{2}{f} - 1 \right) ,
\]  

(4)

where \( a \) and \( b \) are constants depending on the geometry of the capillary and on the pressures \( \rho_1 \) and \( \rho_2 \). For a membrane containing \( N \) capillaries per square centimeter, of arbitrary size and shape, there will be \( N \) equations similar to equation (4), so that

\[
\sum_{i=1}^{N} \dot{n}_i = \frac{1}{\mu T} \sum_{i=1}^{N} a_i + \frac{1}{\sqrt{MT}} \left( \frac{2}{f} - 1 \right) \sum_{i=1}^{N} b_i .
\]  

(5)

The sum on the left hand is related to the volume, \( \dot{V} \), of gas passing through
the membrane per unit time per unit area measured at room temperature $T'$ and atmospheric pressure $p'$, by the relation

$$\sum_{i=1}^{N} b_i = \left( \frac{p'}{RT'} \right)^{\frac{1}{2}} \hat{V}.$$  \hspace{1cm} (6)

Equation (5) may, therefore, finally be written in the form

$$\hat{V} = \frac{A}{\mu T} - \frac{B}{\sqrt{MT}} \left( \frac{2}{f} - 1 \right),$$  \hspace{1cm} (7)

where

$$A = \frac{RT'}{p'} \sum_{i=1}^{N} a_i$$  \hspace{1cm} (8)

and

$$B = \frac{RT'}{p'} \sum_{i=1}^{N} b_i.$$  \hspace{1cm} (9)

Equation (7) not only clearly shows that the permeability of a membrane containing micropores decreases with increasing temperature, but it also provides a method for estimating the mean equivalent diameter of the pores. The method of carrying out the calculation is described in detail in the next section.*

*Estimation of Pore Diameter

For a membrane comprised of a system of $N$ identical cylindrical pores per square centimeter, the expression for $A$ in equation (8) becomes

$$A = \frac{NT'}{p'} \left[ \frac{\pi D^2 (p_2 - p_1) \rho_m}{128 L} \right]$$  \hspace{1cm} (10)

and the expression for $B$ in equation (9) becomes

$$B = \frac{NT'}{p'} \left[ \frac{\pi D^2 (p_2 - p_1) R}{16 L} \left( \frac{2}{f} \right)^{\frac{3}{2}} \right].$$  \hspace{1cm} (11)

The ratio of $B$ to $A$ is then simply

$$\frac{B}{A} = \frac{4\sqrt{2\pi R}}{D\rho_m},$$

and hence the diameter $D$ is given by

$$D = \frac{4A}{B} \frac{\sqrt{2\pi R}}{\rho_m}. \hspace{1cm} (12)$$

*A similar procedure has been used by H. Adzumi, Bull. Chem. Soc. Japan, 1937, 12, 304.
Now if $A/B$ is determined by experiment, $D$ may be calculated. To evaluate $A/B$ we write equation (7) in the form

$$
\frac{\mu T \dot{V}}{T/M} = A + B \left( \frac{2}{f} - 1 \right).
$$

(13)

If then the group $\mu T \dot{V}$ is plotted against the group $\mu \sqrt{T/M}$ for all the gases used with a given shell membrane, a straight line should be obtained, provided $f$ is constant (5). The slope of this line will be the quantity $B \left( \frac{2}{f} - 1 \right)$ and the intercept on the vertical axis the quantity $A$. On Fig. 2, three such curves are shown, one for each shell membrane. All the curves are straight lines, indicating that $f$ is, in fact, a constant within experimental error.

![Fig. 2. Experimental verification of fluid flow law](image)

On the assumption that $f = 1$, $D$ has been calculated for each shell membrane using equation (12). The values 2.4, 2.3, and 2.0 microns have been obtained for shell membranes 1, 2, and 3 respectively. If the pores are not cylindrical, these numbers must be looked upon as equivalent mean diameters. Alternatively, one could use the assumption that the cross-section of each pore is a square and carry out a similar calculation.

We may now go back to equation (11) and calculate the surface density of pores, $N$. In doing so, we are obliged to use the third power of the diameter, $D$, which is only roughly known, and, therefore, the calculated results may be expected to be correct in order of magnitude only. The calculated values for the surface density were $10 \times 10^6$, $15 \times 10^6$, and $40 \times 10^6$ per sq. cm. for shell membranes 1, 2, and 3, respectively. As will be seen below, these values are in good agreement with the electron micrograph data.
The ratio of the second term to the first term of equation (7) indicates the relative rate at which gas is transferred through the membrane by diffusive and viscous flow. If this relative rate is designated by the symbol $G$, we have

$$G = \frac{B}{A} \left( \frac{2}{f} - 1 \right) \rho \sqrt{T/M}.$$ \hspace{1cm} (14)

This equation reveals in a very transparent way the manner in which this relative rate varies with the several experimental variables. In virtue of equation (2), it may be also be written in the form

$$G = \frac{8\lambda_n}{D} \left( \frac{2}{f} - 1 \right),$$ \hspace{1cm} (15)

which discloses that the flow will be predominantly diffusive in character only where the ratio of the mean free path to the mean diameter of the pores is very large. In this situation the second term in equation (7) is the dominant one and Graham's law of diffusion will be accurately true. However, the ratio $\lambda_n/D$ is seldom large at pressures in the neighborhood of 1 atmosphere, and a natural porous membrane obeying Graham's law would be an exceptional one indeed.
The Electron Micrographs

After the above permeability study had been completed, it occurred to the authors that the electron microscope might supply direct evidence of the existence of micropores. Through the kind cooperation of Dr. Harold C. O'Brien of the University of Pittsburgh, we obtained electron micrographs of three different parts of a dried shell membrane. One of these is presented in Fig. 3. The picture is that of an actual dried shell membrane and not of a plastic replica. The white areas designate the holes in the membrane, and, while they vary considerably in size, one can deduce from the scale line that they are actually in the neighborhood of 1 micron. The area covered by each electron micrograph was about 150 X 10^-8 sq. cm. and the number of holes in each picture was about 30. Hence the surface density of holes, N, was about twenty millions.

One can deduce from the electron micrographs that the holes are gaps in a loosely woven fibrous lattice, the individual fibers being again of the order of 1 micron in diameter. The fibers have been shown from chemical studies to consist of a pure typical keratin (6).

CONCLUSIONS

The results obtained in the present work bring out clearly that the dried shell membrane is an open lattice-like network containing micropores of the order of 1 micron in mean equivalent diameter and whose surface density is roughly 20 millions per sq. cm. The gas transfer through this network is largely by viscous flow and accordingly does not obey Graham's law of diffusion.

The data make clear that dried shell membranes are extremely permeable to all the gases examined and that wet membranes are completely impermeable. This fact leads one to suspect that during embryonic development, the rate of influx of gases is controlled in some measure by the moisture content of the shell membrane.

The permeability technique developed here should be of value in the study of other membranes and especially natural membranes of biological importance.

LITERATURE CITED