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The failure of hydrodynamic analysis to define pore size in cell membranes

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The equivalent pore theory predicts that the size of water transporting pores can be calculated from the ratio of osmotic (P_f, cm · s^{-1}) to diffusive (P_d, cm · s^{-1}) water permeability. Determinations of P_f and P_d in human red cells within the last thirty years have increased the ratio of P_f to P_d. According to the equivalent pore theory the pore diameter has increased from 9 Å to 25 Å. A pore diameter of 25 Å is not compatible with the permeability characteristics of the red cell membrane. We conclude that the equivalent pore theory fails to determine pore size in red blood cells. We suggest that water transporting pores in human red cells transport water molecules in a single file fashion.

By the end of the 19th century it had been established that the permeability of cell membranes to solutes increased with the lipid solubility of the substances, as described by Overton's rule [1]. However, since the permeability of the cell membrane to water and a few small hydrophilic solutes was much larger than would be predicted by this lipid solubility concept, it was assumed that an aqueous (hydrophilic) transmembrane pathway also existed and accounted for the observed higher permeability.

In 1957 Paganelli and Solomon [2] following a previous analysis of multicellular membrane permeability [3], employed hydrodynamic principles to provide evidence that transmembrane aqueous pores exist in the red blodd cell and to calculate an effective size for such pores. This approach, which has become known as the 'equivalent pore theory', is based on the difference between viscous and diffusive flows of water through porous membranes. Viscous (bulk) fluid flow through a cylindrical pipe or collection of pores, as in a porous membrane, is driven by the difference in pressure between the two ends of the pipe or pores and is described by the Poiseuille equation. In this equation, the flow is proportional to the radius of the pore raised to the forth power (r^4). On the other hand, the diffusive flow of water caused by the Brownian motion of water molecules is described by Fick's first law of diffusion. Here the diffusive flow is related to the area available for diffusion through the tube or pore (πr^2) and therefore is proportional to the square of the radius. Using the Fick and Poiseuille equations and expression can be derived for the average pore radius (r) of a porous membrane:

$$r = \left(\left(P_f / P_d \right) K \right)^{-2} \quad (1)$$

where P_f is the Poiseuille or bulk flow permeability, P_d is the diffusive permeability and K is a collection of physical constants. This basic equation was slightly modified [3] to account for the steric constraints on the movement of a water molecule into a pore which has a size near its own.

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Subsequently, from measurements of P_d, determined by following the rate of tritiated water exchange in erythrocytes, and P_f, made by following the rate of cell volume change (water flow) induced by mixing cells with a solution of a different osmotic pressure, Paganelli and Solomon [2] calculated the equivalent pore radius of human erythrocytes to be $3.5 \cdot 10^{-8}$ cm. While the authors originally cautioned against a literal interpretation of this pore size, it appeared consistant with the known permeabilities of the red cell membrane to a number of solutes. Water, urea and glycerol which have molecular diameters smaller than the calculated equivalent pore diameter of about $7 \cdot 10^{-8}$ cm were known to penetrate the membrane rapidly whereas sucrose, with a molecular diameter of about $10 \cdot 10^{-8}$ cm, did not penetrate the red cell membrane. Largely because of the good agreement between permeabilities predicted by the 'equivalent pore' and those which were observed experimentally in red cells the temptation to consider the 'equivalent pore' as a real structure became great. Numerous studies have subsequently been carried out in attempts to define the detailed nature of these pores and to establish the chemical characteristics of the solutes which move through them as opposed to those which penetrate cell membranes through lipophilic pathways.

Initially the equivalent pore theory, as formally presented by Solomon [4], was questioned on both theoretical and experimental grounds. The fundamental theoretical concern was that the Poiseuille equation is macroscopic in nature and results from differences in the velocity of the fluid across the cylinder through which the flow is taking place. A pore of $7 \cdot 10^{-8}$ cm would provide a diameter sufficient for only two water molecules to move through the pore side by side. It was reasoned that in such small pores, all molecules would experience identical frictional interactions with the pore walls, no flow velocity difference could exist and consequently the use of the Poiseuille equation was inappropriate. At least a partial answer to this objection came from a computer simulation study [5] of pressure driven flow in a pore whose radius approached the size of the fluid molecule. This simulation predicted that, although laminae of water molecules did not strictly exist in such pores, the average velocity of water molecules across the pores was such that the Poiseuille equation appropriately described the flow of water.

The experimental concern about the equivalent pore analysis arose from the observation that hydrodynamic evidence for pores can be seen in non-porous membranes when solution mixing is inadequate to minimize unstirred layers at the surface of the membrane. In such cases the unstirred layers of fluid adjacent to the membrane provide an additional barrier for diffusion but generally have little effect on the osmotic flow of water across the membrane. The result is to decrease the apparent diffusive permeability of the membrane relative to the osmotic permeability and thereby provide hydrodynamic evidence for pores which do not exist. However, under the experimental conditions used in red cell experiments it has been shown that the unstirred layers are too small to generate the large difference between P_f and P_d [6,7].

Experiments within recent years have shown that most, if not all of the small hydrophilic solutes, such as glycerol and urea which were thought to move predominantly through the aqueous pores are in reality carried by special transport systems. This leaves water as one of the few, if not the only substance to use the pores and emphasizes its importance in characterizing their transport function. furthermore, although there has continued to be less than complete satisfaction with the theoretical basis of the equivalent pore model it has regained much of its lost appeal and a ratio of P_f to P_d greater than 1 is generally considered to be sufficient evidence that aqueous pores exist in a membrane. It has also repeatedly been inferred that the magnitude of the P_f to P_d ratio can be used to calculate the diameter of these pores in the red cell membrane [4,8].

Not only has the equivalent pore continued to grow in popularity but, unfortunately for the theory, also in absolute size. In Fig. 1 it can be seen that as the years have passed and the techniques for determining water permeability of human red cells have improved, the measured value of P_f has nearly doubled while the diffusive water permeability (P_d) as measured by both nuclear magnetic resonance (NMR) methods and radio tracer diffusion has decreased by a factor of two. These changes have the effect of increasing the P_f to P_d

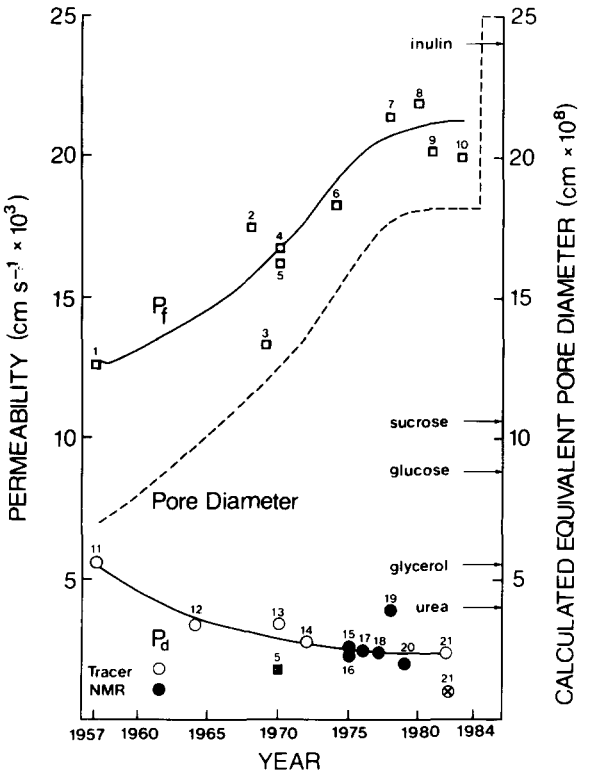

Fig. 1. The 'growth' of the 'equivalent pore' in human erythrocytes with time. The left-hand ordinate shows the osmotic (P_f) an diffusive (P_d) water permeability of the red cell membrane. According to the 'equivalent pore theory' the ratio of P_f to P_d is directly related to the pore size (dashed line) which is shown on the right-hand ordinate. The right-hand ordinate also shows the molecular diameter of several solutes. Determinations of P_f and P_d within recent years that are tabulated elsewhere [13] have increased the P_f to P_d ratio to a magnitude which by using the 'equivalent pore theory' gives a pore size that is inconsistent with both morphological and functional characteristics of the red cell membrane. 1, Sidel and Solomon [14]; 2, Rich et al. [15]; 3, Sirs [16]; 4, Blum and Forster [17]; 5, Macey and Farmer [9]; 6, Colombe and Macey [18]; 7, Galey [19]; 8, Levin et al. [20]; 9, Terwillinger and Solomon [21]; 10, Mlekoday et al. [22]; 11, Paganelli and Solomon [2]; 12, Barton and Brown [23]; 13, Vieira et al. [24]; 14, Macey et al. [25]; 15, Shporer and Civan [26]; 16, Fabry and Eisenstadt [27]; 17, Andrasko [28]; 18, Chien and Macey [29]; 19, Conlon and Outhred [30]; 20, Pirkle et al. [31]; 21, Brahm [10].

ratio and causing a 'growth' in the equivalent pore diameter to a value of about $18 \cdot 10^{-8}$ cm. If the membrane pores were of this size, sucrose and raffinose, to which red cells are impermeable, should easily penetrate the cell membrane.

A further blow to the theory of equivalent pores comes from studies of red cells treated with PCMBS (*p*-chloromecuribenzosulfonate) [9,10]. This agent inhibits both osmotic (P_f) and diffusive (P_d) water permeability to a value of about $1.4 \cdot 10^{-8}$ cm $\cdot$ s^{-1} (see crossed square and circle in the figure). This is very nearly the permeability of non-porous lipid bilayers to water and is generally agreed to represent the permeability of the non-porous lipid moiety of the red cell membranes. Since this permeability pathway is not through pores its contribution should be subtracted from the total membrane permeabilities (both P_f and P_d). When this is done, the equivalent pore diameter is calculated to be $25 \cdot 10^{-8}$ cm. If such pores existed even the 5500 molecular weight inulin molecule should diffuse across the cell membrane. Furthermore, if pores of this average size did exist they should be visible in transmission or scanning electron microscopic views of red cell membranes. Since neither solute permeability nor morphological characteristics provide support for large diameter pores it must be concluded that the equivalent pore interpretation of the difference between the osmotic water permeability and diffusive water permeability is incorrect.

If the equivalent pore theory does not explain the difference between P_f and P_d how can it be interpreted? Although as yet little evidence has been accumulated in support of it, a possible explanation may come from studies of gramicidin A, a polypeptide antibiotic with a known molecular structure. It is generally agreed that this drug forms a continuous transmembrane channel with a diameter of about $4 \cdot 10^{-8}$ cm. Water, but not urea, has been shown to move through these pores and due to their molecular dimensions must do so in a single file fashion. It is interesting that this single file channel shows a P_f to P_d ratio of about 5 and that theoretical analysis of the hydrodynamics as well as measurements of the streaming potential across such pores [11,12] show the P_f to P_d ratio equal to the number of water molecules occupying the pore. It has been suggested that the water-transporting channels of the erythrocyte membrane may be similar single-file pores. If this is so, using the corrected P_f to P_d ratio determined from the figure, we can calculate that the water-transporting channels accomodate 14 to 15 water molecules in a single file. The diameter of the pore

must be at least a little larger than that of a water molecule ($> 3 \cdot 10^{-8}$ cm) and smaller than the diameter of two water molecules ($< 6 \cdot 10^{-8}$ cm) so that the water molecules are constrained to a single file.

Whatever be the correct interpretation of the P_f to P_d ratio it is clear that the equivalent pore theory fails. It is inescapable that the refined measurements of the hydrodynamic parameters from which the equivalent pore size is calculated now lead to a pore diameter which is inconsistant with the known permeability characteristics of the human red cell membrane. Consequently the equivalent pore theory must be abandoned.

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