Oxygen Diffusion in Biological and Artificial Membranes Determined by the Fluorochrome Pyrene

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ABSTRACT Quenching of pyrene fluorescence by oxygen was used to determine oxygen diffusion coefficients in phospholipid dispersions and erythrocyte plasma membranes. The fluorescence intensity and lifetime of pyrene in both artificial and natural membranes decreases about 80% in the presence of 1 atm O_2, while the fluorescence excitation and emission spectra and the absorption spectrum are unaltered. Assuming the oxygen partition coefficient between membrane and aqueous phase to be 4.4, the diffusion coefficients for oxygen at 37°C are 1.51×10^{-5} cm²/s in dimyristoyl lecithin vesicles, 9.32×10^{-6} cm²/s in dipalmitoyl lecithin vesicles, and 7.27×10^{-6} cm²/s in erythrocyte plasma membranes. The heats of activation for oxygen diffusion are low (<3 kcal/degree-mol). A dramatic increase in the diffusion constant occurs at the phase transition of dimyristoyl and dipalmitoyl lecithin, which may result from an increase in either the oxygen diffusion coefficient, partition coefficient, or both. The significance of the change in oxygen diffusion below and above the phase transition for biological membranes is discussed.

Knowledge of oxygen diffusion rates in tissue is essential in evaluation of the role of oxygen in control of metabolism. Oxygen diffusion coefficient in skeletal muscle was found to be 1.3×10^{-5} cm²/s by Krogh (1929). This value closely resembles the oxygen diffusion coefficient in other tissues, and in blood plasma (Hartridge and Roughton, 1927) and erythrocytes suspension (Forster et al., 1957; Klug et al., 1956; Thews, 1968).

The similarity in the oxygen diffusion coefficient between tissue and protein solutions is generally taken to mean that the membrane imposes no barrier for oxygen (Boag, 1969; Wittenberg, 1970). In this paper we attempt critical analysis of this assumption. Quantitative determination of the oxygen diffusion constant is afforded by use of the diffusion-limited reaction between the paramagnetic species oxygen and the singlet excited state of a fluorescent probe molecule located in the membrane. Pyrene is chosen as probe because its in-

trinsic fluorescent lifetime is long, thus collisions between excited state pyrene and oxygen cause significant quenching of fluorescence. Furthermore, pyrene is a lipophilic hydrocarbon which readily partitions into the lipid phase of the membrane, and molecular pyrene diffusion has been previously described (Vanderkooi and Callis, 1974). Derivatives of pyrene have been successfully used to determine the presence of oxygen in proteins (Vaughan and Weber, 1970) and in rat liver cells (Knopp and Longmuir, 1972).

MATERIALS AND METHODS

Chemicals

Pyrene (Eastman Chemical Company, Rochester, N. Y.) was twice recrystallized from ethanol and was determined to be free of fluorescence impurities by a single exponential fluorescence decay over three log units in ethanol. Sigma Chemical Company (St. Louis, Mo.) supplied DL-α-dipalmitoyl lecithin (lot no. 93C-2900) and cholesterol (lot no. 106B-1630). L-α-dimyristoyl lecithin (lot no. 9434) was supplied by Nutritional Biochemical Corporation (Cleveland, Ohio). Prepurified oxygen and nitrogen were obtained from Air Reduction Company (New York). All other reagents were of the highest purity commercially available. Twice-glass-distilled water was used throughout.

Preparation of Membranes

Artificial phospholipid membranes were prepared by sonicating the lecithin and pyrene in phosphate-buffered aqueous solution (pH 7.4) at around 45°C for 3–5 min on a Branson sonifier (Branson Sonic Power, Danbury, Conn.). Erythrocyte plasma membranes from human blood were prepared by the procedure of Dodge et al. (1963), and the protein concentration was determined by the biuret method (Gornall et al., 1949).

Pyrene was incorporated by adding a concentrated alcoholic pyrene solution to aqueous suspension of membrane. The fluorsecence intensity of the pyrene-membrane samples was stable 30 s after pyrene addition, indicating that the partitioning of pyrene into the membrane phase occurred within this time.

Fluorescence Measurements

Steady-state fluorescence was measured with an Hitachi MPF-2A fluorescence spectrometer (Hitachi Ltd., Tokyo, Japan) using conventional 90° optics. The sample was maintained at the desired temperature with the use of circulating water through the cell block. Relative quantum yields of the samples were determined using 386 nm as emission wavelength and 335 nm as excitation wavelength.

The decay of fluorescence was measured on an Ortec photon-counting fluorescent lifetime instrument (EGG. Ortec, Oak Ridge, Tenn.), as previously described (Vanderkooi and Callis, 1974). Temperature control was achieved using a thermoelectric module (Temptronix Company, Needham, Mass.) with thermostatic control incorporated into the sample cell block. Data were analyzed for exponential decay by

nonlinear regression on a PDP-10 computer, also as described previously (Vanerdkooi et al., 1974).

The fluorescence measurements were made using a cuvette with a screw-type cap equipped with a rubber septum (Hamilton Company, Reno, Nev. type 18288) through which a syringe could be inserted to allow addition of gas. Nitrogen and oxygen in the desired ratios were mixed before bubbling by use of a Matheson gas mixer, model no. 7322 (Matheson Co., Inc., East Rutherford, N.J.). The suspensions (3–4 ml) were equilibrated with each gas mixture by bubbling the gas through the suspension for 15 min at a rapid rate, followed by removal of the syringe. Longer times of bubbling did not change the fluorescence lifetime or yield, indicating that equilibration had occurred. The fluorescent lifetimes were constant over a period of 6 hr, which was taken to mean that leakage of the gases to the atmosphere did not occur. The oxygen concentration in the medium was calculated by use of literature values of oxygen solubility in aqueous solutions (International Critical Tables). Since the rate of reaction between excited-state pyrene and oxygen is first order with respect to oxygen concentration (Eq. 4) it is necessary to know the oxygen concentration in the membrane in order to calculate the diffusion constant. In our experiments this value is unknown, and we have calculated the diffusion coefficient assuming oxygen to be in the same concentration in the membrane as in the aqueous medium. A more reasonable estimate of oxygen concentration is to use the distribution coefficient for membrane/water of 4.4, determined by Battino et al. (1968), and using this value, the diffusion coefficient was also calculated.

In all experiments, pyrene concentration was kept low so that pyrene excimers were not formed (Vanderkooi and Callis, 1974). The absence of excimers was confirmed by the absence of fluorescence emission at 470 nm. This precaution is necessary since excimer formation competes with oxygen for the quenching of excited-state pyrene monomer, thus complicating analysis.

CALCULATION OF DATA

The relevant processes involved in determination of diffusion constants from fluorescence quenching data are given below:

$$P + h\nu \rightarrow P^* \text{ (absorption of light)}, \tag{1}$$

$$P^* \xrightarrow{k_f} P + h\nu' \text{ (fluorescence emission)}, \tag{2}$$

$$P^* + O_2 \xrightarrow{k} \text{(quenching)}, \tag{3}$$

where P and P^* represent the respective ground and excited states of the fluorescent molecule, i.e., pyrene, and k_f is the rate of fluorescence decay, which is related to fluorescence lifetime, τ by

$$k_f = \frac{1}{\tau}. \tag{4}$$

The biomolecular rate constant, k, for O_2 quenching of pyrene fluorescence can be obtained from the Stern-Volmer Equation (Stern and Volmer, 1919):

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + k\tau_0[O_2], \tag{5}$$

where F_o and τ_o are the fluorescent intensities and lifetimes in the absence of quencher and F and τ are the intensities and lifetimes in the presence of quencher. Making the assumption used by others (Osborn and Porter, 1965; Lakowicz and Weber, 1973) that every collision between P^* and oxygen is effective in quenching, the rate constant, k, is then related to the diffusion constant, D, by the Einstein-Smoluchowski expression:

$$k = 4\pi RDN', \tag{6}$$

where N' is Avogadro's number per millimole, R is the sum of the molecular radii, and D is formally the sum of the diffusion coefficients for oxygen and pyrene. In our calculations we assumed that D is the diffusion coefficient of oxygen alone, a reasonable assumption on the basis that the diffusion coefficient of oxygen is much larger than for pyrene. We further assumed that the interaction radius, R, is 6×10^{-8} cm, an assumption which is based on the molecular sizes of pyrene and oxygen.

Since the fluorescence yields and lifetimes are related to the rate, k, (Eq. 4) the heat of activation (E_a) for oxygen diffusion can be calculated by two methods, Arrhenius plots of fluorescence intensity (F) or fluorescence lifetime (τ) versus temperature:

$$\log\left(\frac{F_0}{F}\right) = \frac{E_a}{2.303RT} + C_1, \tag{7}$$

or

$$\log\left(\frac{\tau_0}{\tau}\right) = \frac{E_a}{2.303RT} + C_2, \tag{8}$$

where T is the absolute temperature, R is the gas constant, and C_1 and C_2 are constants. Both these methods were used to determine E_a as a check for the internal consistency of the measurements.

RESULTS

Fluorescence Emission Parameters of Pyrene

The fluorescence yield of pyrene incorporated into phospholipid vesicles is quenched by either complex formation between ground-state pyrene and oxygen, (Evans, 1953) or alternatively, between excited-state pyrene and oxygen as described in Eqs. 1–3. That the second mechanism accounts for the fluorescence quenching is supported by several lines of evidence: (*a*) The absorption spectrum of pyrene in ethanol is unaltered by the presence of atmospheric

O_2. (*b*) The fluorescent lifetime, τ, is decreased by the presence of oxygen and the decay is exponential, as seen by the linear relationship between the logarithm of fluorescence intensity and time (Fig. 1). (*c*) The fluorescent yield of pyrene but not its excitation and emission spectra is affected by the presence of oxygen in the sample. These phenomena are in agreement with the reaction mechanism given in Eqs. 1–3 and with the Einstein-Smoluchowski equation for diffusion given in Eq. 6.

Diffusion Coefficient for Oxygen in Membranes

The fluorescent lifetimes for pyrene were measured in different phospholipid vesicles or membranes as a function of O_2 concentration and temperature (Table I). The lifetime of pyrene in the absence of the quencher, oxygen, was around 200 nl at 25°C for all membranes, and as the temperature was raised, the lifetime decreased.

These data are plotted according to the Stern-Volmer relationship (Eq. 4) in Fig. 2. The slope of these plots yields the rate constant for the reaction of oxygen with pyrene, and allows calculation of diffusion coefficients (Eq. 5). A summary of the results is presented by Table II. The diffusion constant is presented assuming oxygen concentration in the membrane to be the same as in the aqueous medium (third column) or more realistically to be more concentrated in the membrane, (last column). The diffusion coefficient of oxygen in phospholipids agrees closely with that in red blood cell membranes, and is close to literature values in water or tissue. This finding suggests that the diffusion

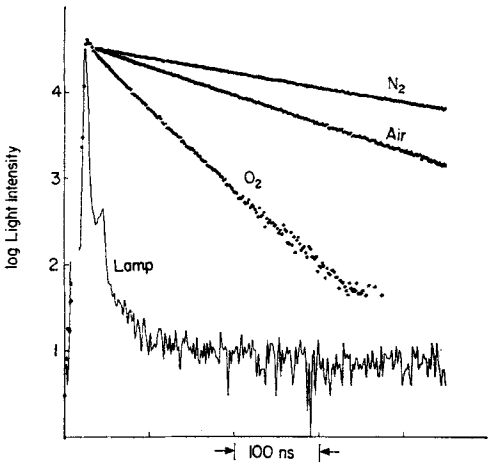

FIGURE 1. Fluorescence decay of pyrene in dimyristoyl lecithin vesicles. The samples contained 0.3 mg dimyristoyl lecithin/ml, 0.1 μM pyrene, and 10 mM PO_4 buffer, pH 7.4. The samples were equilibrated with gases indicated on the figure, and fluorescence decay curves were obtained as described in Materials and Methods. Temperature was 25°C.

TABLE I

FLUORESCENT LIFETIMES AS A FUNCTION OF LIPID COMPOSITION, OXYGEN CONCENTRATION, AND TEMPERATURE

Membrane type	Temperature	Oxygen concentration				
		0%	21%	50%	75%	100%
	°C	*ns*	*ns*	*ns*	*ns*	*ns*
Dimyristoyl lecithin	25	200	119	56	45	35
	28	197	101	50	46	35
	37	178	87	48	36	30
	45	145	58	38	32	32
Dipalmitoyl lecithin	25	230	102	79	85	60
	28	190	96	85	71	56
	37	163	88	41	42	41
	45	137	74	42	35	29
Erythrocyte plasma membrane	25	188	108	84	73	68
	28	180	109	82	62	59
	37	166	94	75	56	49
	45	141	88	67	45	40
Dimyristoyl lecithin cholesterol (3:1)	25	224	123	83	64	50
	28	205	114	79	58	47
	37	194	83	63	50	40
	45	155	80	58	53	33

Conditions as in Fig. 2.

barrier imposed by the membrane is not large. It is further interesting to note that the oxygen diffusion coefficient for the gas phase is only one to two orders of magnitude larger than that found for tissue or membranes.

Heats of Activation for Oxygen Diffusion

The temperature dependence of oxygen diffusion can be measured from steady-state measurements of fluorescence intensity (Eq. 6) or from measurements of the decay of fluorescence (Eq. 7). The Arrhenius plots of fluorescence intensity and lifetimes are plotted in Fig. 3. Two things are noteworthy for these data: (*a*) A sudden decrement in fluorescence intensity occurred at 20°C for dimyristoyl lecithin and at 37°C for dipalmitoyl lecithin. These are temperatures at which phase transitions have been shown to occur (Chapman et al., 1967) and the decrease in fluorescence intensity can be accounted for by an increase in the diffusion rate for oxygen upon melting, and/or an increase in oxygen solubility above the phase transition. (*b*) The energy of activation, E_a, is under 3 kcal/degree-mol for oxygen diffusion in all membranes, a feature common to diffusion-limited reactions.

It should be noted that the fluorescence intensity was the same for increasing and decreasing temperatures for each sample, except for the mixed lecithin-

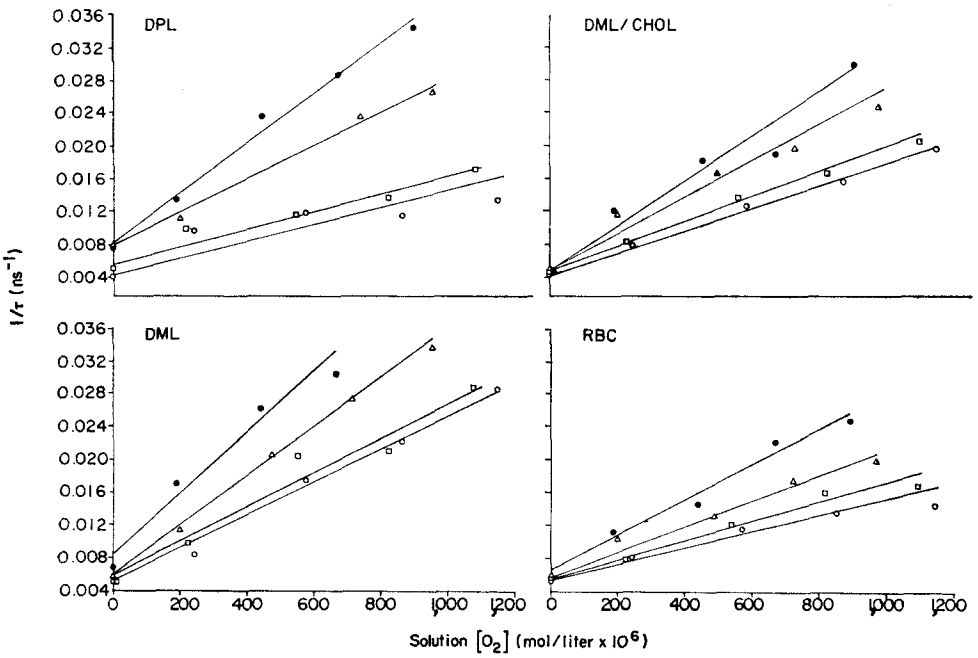

FIGURE 2. Reciprocal fluorescent lifetimes of pyrene as a function of temperature and oxygen concentration in membranes of differing lipid composition. All samples contained 0.5 μM pyrene and 0.1 mM PO_4 buffer, pH 7.4. The dimyristoyl lecithin (DML) and dipalmitoyl lecithin (DPL) samples contained 3.3 mg lecithin/ml. The sample labeled DML/CHOL contained 0.07 mg cholesterol and 0.23 mg dimyristoyl lecithin/ml. Erythrocyte ghost preparations (RBC) contained 0.3 mg protein/ml./○ = 25°C, □ = 28°C, △ = 37°C, ● = 45°C.

cholesterol vesicles. Below 25°C the temperature profile of the Arrhenius plots showed hysteresis (Fig. 3). One explanation for this behavior is that a phase separation between the lecithin and cholesterol occurred similar to that postulated to occur in other membranes containing mixtures of phospholipids (Shimshick and McConnell, 1973).

DISCUSSION

Oxygen diffusion coefficients in biological and artificial membranes were measured making use of a diffusion-limited reaction between excited-state pyrene and oxygen, a reaction which can be monitored by measurement of the quenching of pyrene fluorescence. The diffusion coefficient of oxygen measured by the quenching of pyrene fluorescence is within an order of magnitude of the values measured by other techniques indicating the validity of the approach (Table II). It is, nonetheless, useful to examine assumptions made in calculating the diffusion coefficient by fluorescence-quenching techniques. These assumptions are:

TABLE II

OXYGEN DIFFUSION AND RATE CONSTANTS OF OXYGEN AS A FUNCTION OF TEMPERATURE AND LIPID COMPOSITION

Membrane type	Temperature	k	D	D/4.4	Reference
	°C	$M^{-1}s^{-1}$	cm^2/s	cm^2/s	
Dimyristoyl lecithin	25	21×10^9 (14%)	4.7×10^{-5}	1.1×10^{-5}	*
	28	21 (7.5%)	4.7	1.1	
	37	30 (2%)	6.7	1.5	
	45	38 (14%)	7.9	1.8	
Dipalmitoyl lecithin	25	7×10^9 (24%)	1.56×10^{-5}	3.55×10^{-6}	*
	28	10 (10%)	2.3	5.3	
	37	19 (11%)	4.1	9.3	
	45	31 (5%)	6.8	15.4	
Erythrocyte plasma membrane	25	10×10^9 (31%)	2.2×10^{-5}	5.0×10^{-6}	*
	28	11 (8%)	2.4	5.4	
	37	14 (6%)	3.2	7.3	
	45	22 (6%)	4.9	11.2	
Dimyristoyl lecithin cholesterol (3:1)	25	13×10^9 (5%)	2.9×10^{-5}	6.6×10^{-6}	*
	28	15 (2%)	3.2	7.5	
	37	19 (5%)	4.3	9.7	
	45	24 (11%)	5.3	12.1	
Gas phase	25	1.52×10^{11}	3.36×10^{-4}		‡
30% hemoglobin solution	37		7.1×10^{-6}		§
Saline	25		2.07×10^{-5}		‖
Pepsin	25	2.3×10^9			¶
Muscle	37		1.3×10^{-5}		**

Conditions as in Fig. 2. Numbers in parenthesis refer to confidence intervals at the 50% level using the Students t test.

* This work.

‡ Calculated.

§ Klug et al., 1956.

‖ Goldstick, 1966.

¶ Lakowicz and Weber, 1973.

** Krogh, 1929.

(*a*) The oxygen concentration is known. Since oxygen is a lipophillic substance, its concentration in the hydrocarbon core of the membrane is greater than in the aqueous medium. The distribution coefficient for oxygen between water and lipid has been determined to be 4.4 (Battino et al., 1968), but no precise data are available on whether the distribution changes with altered membrane composition or temperature. Whether the increase in pyrene quenching above the phase transitions of dimyristoyl and dipalmitoyl lecithin is due to increased solubility or an increase in diffusion coefficient cannot be determined at the present time; however, the conclusion is the same: the net flux of oxygen in a membrane in the fluid state will be greater than for a membrane in the paracrystalline state.

(*b*) The interaction radius is known. Oxygen is paramagnetic and is thought to quench fluorescence by catalyzing conversion to triplet state (Parker, 1968). The critical distance for this process is unknown, and may be larger than the value based on consideration of molecular size. If so, the diffusion coefficients are smaller by a factor of perhaps 2 than the values we assigned.

(*c*) The rate of diffusion of pyrene is negligibly small compared to the oxygen diffusion rate. This assumption appears to be valid since independent measurement of pyrene diffusion in membrane gave diffusion coefficients for pyrene two orders of magnitude smaller than for oxygen (Vanderkooi and Callis, 1974).

(*d*) Every collision between excited-state pyrene and oxygen is effective in producing quenching. If the frequency of collisions is not the same as for encounters (ie. reaction), the diffusion coefficient which we calculated will be smaller than the actual diffusion of oxygen (for a discussion, see Glasstone et al., 1941). However, the validity of this assumption is supported by the finding that the heats of activation for oxygen diffusion are low. It is, furthermore, based on the results of others (Osborn and Porter, 1965; Vaughan and Weber, 1970).

(*e*) Oxygen diffusion in the membrane is isotropic. A diagrammatic scheme of the reaction system emphasizing that the membrane represents a heterogeneous and anisotropic medium is presented in Fig. 4. It is apparent that one might expect several rates for oxygen diffusion in such a system: one rate might describe oxygen diffusing laterally *in* the membrane and another rate might govern transverse diffusion *into* the membrane. If these rates were significantly different, the decay of pyrene fluorescence would be described by more than one exponential. However, a single exponential decay of pyrene fluorescence was observed (Fig. 1). This provides evidence that the assumption that the rates of oxygen diffusion into and in the membrane are nearly the same and supports the assumption that oxygen diffusion is isotropic.

The above assumptions are reasonable and the diffusion coefficient calculated by this method is likely to be correct within a factor of about 5. The

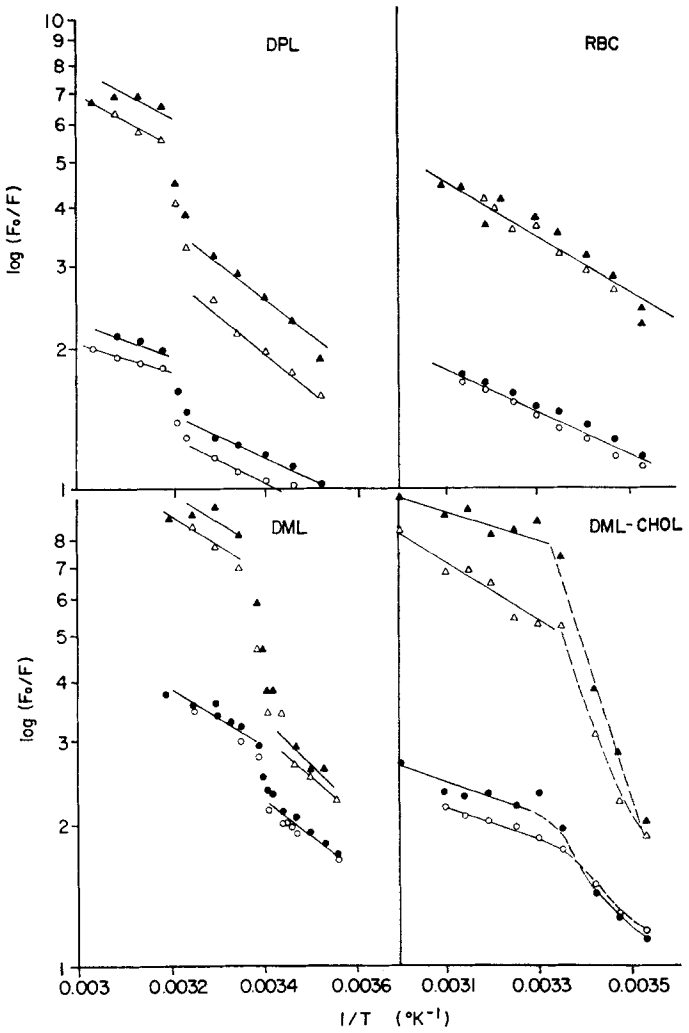

FIGURE 3. Fluorescent intensity of pyrene in membranes of differing lipid composition as a function of temperature and oxygen concentration. All samples are as in previous figure. Open symbols indicate measurements made ascending in temperature; solid symbols, descending. F_o represents relative fluorescence in sample equilibrated with nitrogen and F represents relative fluorescence intensity in identical sample equilibrated with air (○) or oxygen (△).

method is, however, very precise and small differences in rate constants are significant.

It is interesting to note that the oxygen diffusion coefficient in membranes increases by a factor of 3–4 upon melting. Although the differences in oxygen coefficient are not large, in a biological system where metabolism is finely controlled, these differences may be significant. The lung surfactant is com-

TABLE III

HEATS OF ACTIVATION FOR OXYGEN DIFFUSION

Membrane	Temperature range	ΔH_{act}
	°C	*kcal*
Dimyristoyl lecithin	7–21	1.4
	23–40	1.1
Dipalmitoyl lecithin	15–35	1.6
	40–60	1.1
Cholesterol-dimyristoyl lecithin	37–60	1.1
Red blood cell membrane	9–49	1.1

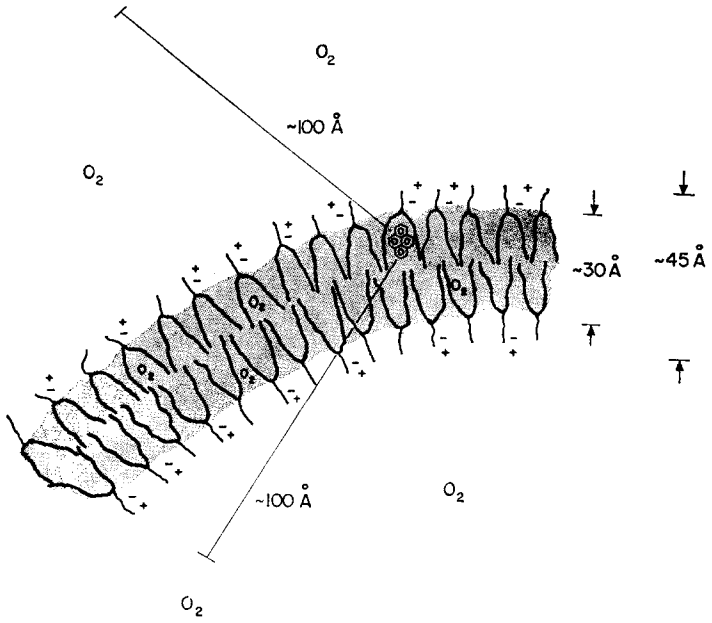

FIGURE 4. Hypothetical membrane.

posed predominantly of L-α-lecithin, (Pattle, 1965) the melting point of which is 41–42°C. Since the lung is bathed in air, under normal conditions the rate of oxygen diffusion across the surfactant and other membranes may not be critical in oxygenation, the chemical rate of combination with hemoglobin being more important. The red blood cell membrane, which contains approximately equimolar ratios of cholesterol and phospholipids (van Deenan and DeGier, 1964), shows an intermediate diffusion coefficient of oxygen compared with the fully melted or crystalline membrane. The cellular and subcellular membranes in tissue such as brain, liver, kidney, and muscle are typically composed of phospholipids containing unsaturated fatty acids, the melting point of which is considerably below physiological temperatures

(White, 1973). Oxygen diffusion in these membranes is favored by the fluidity of the membranes.

Our finding that the diffusion coefficient for oxygen measured in the membrane is about two orders of magnitude larger than for pyrene is significant from a theoretical point of view. The Fick-Einstein theory of diffusion predicts that the diffusion coefficient is inversely proportional to R, the molecular radius, and thus fails to predict the difference in diffusion coefficient. The faster diffusion rate for oxygen can be explained by oxygen's small size in comparison with the molecules of the membrane. Oxygen is expected to experience "holes" between the molecules of the membrane, and consequently its movement is relatively unimpaired and is only about an order of magnitude smaller than in gaseous phase. Pyrene and other larger electron paramagnetic resonance or fluorescent probes are more restricted by the surrounding membrane molecules and thus are more sensitive to medium "viscosity." The finding that the Fick-Einstein equation does not hold for diffusion of small molecules is not surprising since this theory assumes that the medium is homogeneous and continuous, obviously invalid when the diffusant is very small compared with the molecules of the medium. It points to the difficulty of predicting the diffusion rate of one molecule from measurements made on another molecule, and the need to experimentally determine the diffusion coefficients.

An interesting comparison can be made between the rate of oxygen interaction with pyrene located in the membrane, and with cytochrome oxidase, a membrane component of mitochondria. The rate constant for the reaction between pyrene and oxygen ranged from about 0.7 to 3 $\times$ 10^{10} $M^{-1}s^{-1}$ for all membranes tested in the temperature range of 25–45°C. The rate of reaction for cytochrome oxidase, a membrane protein, and oxygen is estimated to be 0.7 to 1.0 $\times$ 10^{7} $M^{-1}s^{-1}$ (Gibson and Greenwood, 1963; Chance and Schindler, 1965). This rate is two to three orders of magnitude slower than the reaction between pyrene and oxygen, and suggests that an orientational constraint may account for the slower reaction of cytochrome oxidase; alternatively, the reaction may not be diffusion limited, the rate depending upon the chemistry of the system.

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