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Dioxygen solubility in aqueous phosphatidylcholine dispersions

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The solubility of molecular oxygen, or dioxygen, in low weight percent (1.5%) sonicated dimyristoylphosphatidylcholine (DMPC) aqueous dispersions saturated with air has been measured as a function of temperature between 10°C and 40°C. A modified Winkler technique was used involving a dual cell coulometric titration with voltammetric endpoint detection in a mixed solvent (methanol/water). The results indicate that dioxygen is approximately four times more soluble in the liquid crystalline bilayers (above 24°C) than in the gel state bilayers (below 24°C). The solubility of dioxygen in the bilayer does not appear to be strongly temperature dependent on either side of the 24°C phase transition. The dioxygen solubility in gel state DMPC is approximately equal to that in water at the same temperature. Our results are contrasted with recent measurements made using EPR spin labels.

Introduction

The solubility of dioxygen in both biomembranes and model phospholipid bilayer dispersions has been studied using a variety of direct (manometric [1]) and indirect (fluorescence [2], phosphorescence [3], NMR [4], and EPR [5–8]) techniques. We have developed a simple and direct method for dissolved dioxygen determination in phospholipid bilayer membrane dispersions as a function of temperature that is based on the classical Winkler titration technique [9,10] modified by us for work with phospholipids.

The Winkler technique is based on the quantitative oxidation of Mn(II) to Mn(III) by dioxygen in alkaline solution with the subsequent oxidation of iodide ion to triiodide by the Mn(III) in acidic solution. Winkler titrated the triiodide formed using thiosulfate and a starch indicator.

In this study, we measure the solubility of dioxygen in a water blank simultaneously with the solubility in a dilute (1.5% w/w) dispersion of phospholipids in water. From the difference in solubility between the blank and the lipid dispersion we calculate the solubility in the lipid phase. This measured difference is small compared to the measured values of dioxygen in the sample or blank. Hence, a very accurate, precise and reproducible methodology had to be developed to obtain the measurements.

The following weaknesses of the classical Winkler method were addressed in this study. (1) The starch indicator is not reliable in the presence of surfactants. (2) The iodine formed in the processing of the sample can be lost due to volatilization. (3) Air oxidation of iodide can occur in the acidified solution during the slow titration. (4) Due to the limited availability of highly purified lipids small sample sizes were required (35 ml versus the 300 ml used in typical water analysis). (5) Thiosulfate is not stable over long periods of time and requires frequent restandardization.

In this study we eliminate iodine volatilization by reducing the triiodide with excess reducing agent and back-titrating the remaining reducing agent with coulometrically generated iodine using a voltammetric endpoint. The sample and blank are titrated simultaneously with the coulometric current passed through both cells in series. By using a methanol/water mixture during the titration we eliminate interference caused by the surfactants at the platinum indicator electrodes. Phenylarsineoxide, PAO, rather than thiosulfate is used as the

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; PAO, phenylarsineoxide.

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reducing agent because it is more stable. A schematic of the reactions and procedure is as follows:

(1) Sample equilibration with thermostatted water saturated air.

(2) Addition of alkaline iodide and Mn^{2+} to sample.

$$Mn^{2+} + 2OH^- \rightarrow Mn(OH)_2$$

$$2\,Mn(OH)_2 + \tfrac{1}{2}O_2 + H_2O \rightarrow 2Mn(OH)_3$$

(3) Acidification by concentrated phosphoric acid.

$$2\,Mn(OH)_3 + 6\,H^+ + 3\,I^- \rightarrow 2\,Mn^{2+} + I_3^- + 6\,H_2O$$

$$I_3^- \leftrightarrow I_2 + I^-$$

(4) Addition of excess PAO.

$$I_3^- + C_6H_5AsO + 2\,H_2O \rightarrow 4\,H^+ + 3\,I^- + C_6H_5AsO_3^{2-}$$

(5) Voltammetric titration in methanol/water solution (1/1 by volume) where the excess PAO is oxidized by electrogenerated I_2.

A special sample bottle and protocol, described in the experimental section, was developed to minimize loss due to volatilization of I_2 and air oxidation of I^- during the titration.

Experimental

Sample bottle. The sample bottle shown in Fig. 1 is calibrated for net volume while containing one glass covered stirbar and with the 14/20 standard taper displacement stopper inserted. The volume of the bottle is about 35 ml. The annular region immediately above the assembled joint and adjacent to the displacement stopper arm is the chemical reservoir. Glass coated magnetic stirbars are used since Teflon can absorb significant quantities of dioxygen.

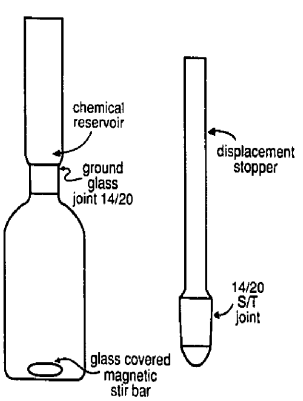

Fig. 1. Glass sample bottle and displacement stopper. When assembled a definate sample volume of about 35 ml is enclosed. The term 'chemical resevoir' in the text refers to the anular volume above the ground glass joint between the two parts of the apparatus.

Sample preparation. Approx. 0.5 g of DMPC lipid (Sigma Chemical Co.) is dried in vacuo and weighed into the sample bottle. A sodium azide solution (3.0 mM) prepared from glass distilled Milli-Q water is added to a level of about one centimeter below the ground glass joint. The lipid sample is then sonicated in a 40 watt bath type sonicator (Ultrasonic Devices) for 6 h. Distilled water is added to a level of about 2 mm above the bottom of the ground glass joint. The sample is placed in a stirred water bath inside a double-walled enclosed chamber. Thermostated water is recirculated in the space between the double walls. The stirred sample is equilibrated in this chamber with water saturated filtered air. After equilibration, (at least 24 h of stirring), 0.25 ml of alkaline iodide reagent is added well below the surface of the sample. This is followed by 0.25 ml of a concentrated $MnSO_4$ solution (364 g $MnSO_4 \cdot H_2O$/l) dispensed in the same way. The displacement stopper is immediately placed into the sample container displacing about 0.3 ml of sample. The displaced sample is carefully removed from the sample bottle chemical reservoir with a pasteur pipet. This displaced lipid sample is dried and then weighed in order to make a correction for the weight of the lipid in the final sample. The samples are then stirred in the constant temperature bath for about 5 min to ensure that all the O_2 is converted to an equivalent amount of $Mn(OH)_3$. The stirring is stopped and the $Mn(OH)_3$ precipitate is allowed to settle. The glass displacement stopper is then removed from the sample bottle with care not to disturb the $Mn(OH)_3$ precipitate, and 0.5 ml of concentrated H_3PO_4 is added below the sample surface. The displacement stopper is replaced and the sample is stirred to ensure stoichiometric generation of I_2. A known volume (6–7 ml) of PAO (0.0182 M) is added to the reservoir of the sample bottle. The displacement stopper is carefully removed and rinsed into the sample bottle, layering the PAO over the analyte. The sample is then stirred slowly in such way that the yellow iodine solution never makes contact with the upper surface of the PAO solution, thus eliminating the possibility of loss of iodine due to volatilization. The sample and a NaN_3 containing blank are each decanted and rinsed into titration cells which have been previously charged with 100 ml of reagent grade methanol and two grams of KI, (required for the coulometric generation of iodine). Argon gas is passed into and over the cells through a sidearm tube. The titration cell overhead stirrers are turned on and the back-titration of the excess PAO in both the sample and blank cells in series is begun. The stirring rate is optimized to prevent excessive foaming of the solution during the titration.

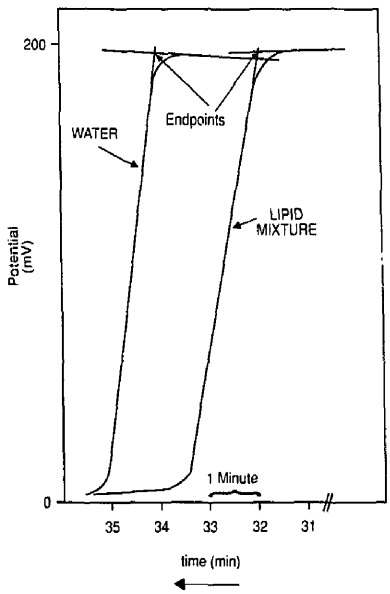

Fig. 2. Typical titration endpoints. Dual channel recorder tracing of the endpoint region of the sample (lipid) and blank (water) coulometric titrations measured at a common electrolysis current. Detection for each sample is potentiometric at a constant one microampere detection current between platinum microelectrodes.

Titration apparatus. The excess PAO in both the sample and blank is titrated simultaneously with electrometrically formed I_2. A constant current (2.9540 ± 0.0002 mA) is passed through the two cells in series to insure that the sample and the blank are subjected to the same current. I_2 evolves at the 20 cm^2 platinum gauze anodes and H_2 evolves at the carbon rod cathodes. A fritted glass disc serves as a salt bridge and prevents the PAO from entering the cathode chambers.

The end points are observed using two electrode potentiometry at constant detector current [11]. A pair of polarizable platinum microelectrodes (4 mm^2) is placed in the anode chamber of each cell. Each microelectrode pair is connected to a one microampere current supply (a 9 V battery in series with a 10 megohm resistor). The potential required to maintain this current across these indicator electrodes is simultaneously monitored in each cell using a HP Model 7128A dual pen recorder. The electrolysis circuit and the endpoint indicating circuits must not have a common ground. The recorder tracings are used for endpoint detection. Beyond the equivalence point the potential across the indicator electrodes drops precipitously due to excess I_2. The straight line regions before and after the endpoint are extrapolated to yield the equivalence point at their intersection. An example of a pair of endpoints from the titration of a lipid containing sample and an aqueous blank are shown in Fig. 2.

Results

The solubility parameter used is the Bunsen coefficient, α, which is defined as the ratio of the volume of gas (reduced to 0°C and 760 torr) which, at the temperature of measurement, is dissolved in one volume of the solvent. The blank sample is used to determine the Bunsen coefficient of dioxygen in water (α_w) Our values for α_w between 15 and 45°C are given by the following power series in the temperature, t:

$$10^3\alpha_w = 44.2728 - 0.7957 \cdot t + 0.0067 \cdot t^2$$

We also determine the Bunsen coefficient for dioxygen in the entire lipid containing sample (α_s). The Bunsen coefficient for dioxygen in the lipid bilayer, α_b, can be calculated from the following equation:

$$\alpha_s = X_w\alpha_w + X_b\alpha_b$$

where X_w is the volume fraction of water:

$$X_w = \{V_S - M_L/D_L\}/V_S$$

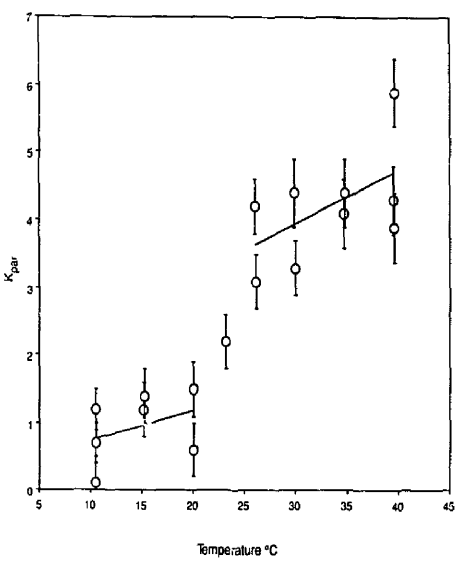

Fig. 3. Dioxygen partition coefficients vs. temperature. Partition coefficients (concentration ratio) are for dioxygen between the DMPC bilayer membrane (1.5%, w/w) and the surrounding water. The solubility of dioxygen in the membrane increases by a factor of 3 or 4 above the gel to liquid-crystalline phase transition (23–24°C) of the lipid bilayers.

and X_b is the volume fraction of the lipid in the sample:

$$X_b = \{M_L/D_L\}/V_S$$

V_S is the total volume of the sample and M_L is the mass of the lipid. The density of the DMPC bilayers, D_L, has been determined to be approx. 1.03 g/ml at 25°C [12].

The partition coefficient, K_{par}, for dioxygen between the bilayer phase and water is calculated from the ratio of the Bunsen coefficients:

$$K_{par} = \alpha_b/\alpha_w$$

In Fig. 3 the partition coefficient for dioxygen between the bilayer phase and water is plotted as a function of temperature. The temperature dependence of K_{par} (away from the lipid phase transition temperature) is due largely to the decreased solubility of dioxygen in water with increasing temperature.

Discussion

Some of the advantages of our methodology of dioxygen measurement are:

(1) The method is accurate. Using the above equation for α_w we are able to reproduce accepted literature values for the solubility of dioxygen in fresh water between 15 and 45°C to within 3 ppt [10].

(2) Our method uses a smaller sample volume (35 ml) than is ordinarily used in Winkler dioxygen determinations. This makes application possible to some biological systems where sample size is limited.

(3) The small weight percentage of phospholipid in our samples, 1.5%, allows us to approximate the dioxygen solubility of the aqueous phase by the solubility of dioxygen in water. In studies with a large weight fraction of lipid a significant amount of the aqueous phase consists of non-solvent water. It has been estimated by Katz and Diamond [13] that ten molecules of water are required to solvate the head group of each DMPC molecule. These ten molecules of head group water were shown to be unavailable for solvation of sucrose. Similarly, it is reasonable to suppose that water bound to the head group will not solvate dioxygen to the same degree as bulk water. From the data presented in the work by Subczynski and Hyde [5] we calculate that about 45% of the water content of their sample is head group water. This may explain why our values for dioxygen solubility in bilayer membranes do not agree with theirs.

(4) Of the available methods for determining dioxygen solubility in bilayer membranes our method has the advantage of being a simple chemical determination. The results do not depend on assumptions about spectroscopic parameters.

The chemical stability of the lipids bathed in oxygen is a potential problem. We attempted to extend this study (F.T. Moy, M.S. thesis, San Francisco State University, 1987) to include mixed bilayers containing cholesterol and DMPC to study the effect of the sterol on the oxygen solubility. We were unable to obtain consistent dioxygen solubility results with this binary lipid system. We believe that the sterol is subject to chemical attack under the experimental conditions. Substitution of the saturated cholesterol analog dihydrocholesterol in place of cholesterol was to no avail. In addition we noted that after equilibration with air each sample had a characteristic odor not present in the freshly prepared lipid dispersion. We suspect that the sterol molecules were attacked by oxygen radicals present, in spite of the use of glass distilled water to prepare the samples. Evidently the binary lipid system was more susceptible to this attack than the pure DMPC sample. The attack of oxygen free radicals on lipids and other organic molecules is well documented [14,15]. In earlier work in this laboratory we attempted to use this modified Winkler technique to study dioxygen solubility in DMPC bilayer membranes at one atmosphere partial dioxygen pressure (E.S. Smotkin, M.S. thesis, San Francisco State University, 1985). These experiments resulted in unacceptable experimental scatter, which may have been due to lipid oxidation at an enhanced rate due to the large dioxygen partial pressure. It is also interesting to note that Subczynski and Hyde [5] reported that their initial experiments in a brass sample container were unsuccessful due to 'oxygen consumption'. It may be that their brass apparatus gave rise to copper ions which are known to catalyze the Fenton reaction resulting in oxy radicals [16].

Conclusions

The results indicate that dioxygen is three to four times more soluble in the liquid crystalline state (above 24°C) than in the gel state (below 24°C). The dioxygen solubility in gel state DMPC is approximately equal to that in water at the same temperature. Except for the solubility increase noted at the 24°C phase transition, the solubility of dioxygen in the bilayer does not appear to be strongly temperature dependent.

In a previous report from this laboratory Windrem and Plachy [17] reported on the use of EPR with doxyl fatty acid spin probes in dialkyl lecithin bilayers to obtain the product of the dioxygen Bunsen coefficient, $\alpha(O_2)$, times the dioxygen diffusion coefficient, $D(O_2)$. By using a family of doxyl fatty acids they were able to demonstrate that this product is a function of position in the bilayer, rising to a maximum at mid-bilayer. Similar results have been recently obtained by Subczynski and Hyde [8] using the same family of spin probes. These workers also observed that cholesterol causes a

dramatic decrease in the dioxygen diffusion-solubility product. Fischkoff and Vanderkooi [2] had earlier obtained similar results for the $D(O_2)\alpha(O_2)$ product in phospholipid bilayers using the effect of dioxygen on the lifetime of a pyrene fluorescence probe dissolved in a bilayer membrane.

The $D(O_2)\alpha(O_2)$ product is of considerable interest because of its importance in determining the dioxygen transport rate through membranes. The dioxygen Bunsen coefficients determined above now allow us to factor this product and thus obtain an average value for the dioxygen diffusion coefficient, $D(O_2)$, in the bilayer membrane. In this manner we have determined that $D(O_2)$ in DMPC is about $1.0 \cdot 10^{-5}$ cm^2/s at 30°C increasing to $1.2 \cdot 10^{-5}$ cm^2/s at 40°C. These values agree with earlier estimates by Fischkoff and Vanderkooi [2] based on an assumed dioxygen solubility in bilayer membranes by analogy to the measured dioxygen solubility in olive oil [18]. This large diffusion coefficient should be considered an estimate of the in plane components of the anisotropic diffusion tensor of dioxygen in the hydrocarbon region of the bilayer. The component of this tensor normal to the bilayer plane is likely to be smaller due to the high diffusion resistance of the crystalline DMPC headgroup.

We have attempted to reconcile our results with the results of Subczynski and Hyde [5]. The dioxygen partition coefficients that we report in Fig. 3 cannot be directly compared to those in Reference 5, since their values are the dioxygen partition coefficients of the alkyl chain region of the bilayer relative to bulk water, K'_{par}. The calculation of K'_{par} from measured K_{par} values requires both an assumption of the solubility of dioxygen in the bilayer head group region, and an estimate of the fraction of the bilayer volume occupied by the head group. In the calculations below this head group volume fraction is assumed to be 0.33 for DMPC [12]. Subczynski and Hyde make an arbitrary assumption that the DMPC head group dissolves dioxygen to the same extent as does bulk water. When we recalculate our results using this same assumption, we find that $K'_{par} \sim 1.38\ K_{par}$ for all temperature points above the main phase transition of the DMPC. Thus Subczynski and Hyde report $K'_{par} \approx 2.7$ at 26°C and 3.5 at 40°C, while from our data we calculate these values to be 4.9 and 6.6, respectively. Therefore, with a consistent set of assumptions, we find at least 80% more dioxygen dissolved in the hydrocarbon chain region of the bilayer membrane.

An alternate assumption about the head group region is that essentially no dioxygen will be found in this region. Our reasons for this suggestion are (1) that dioxygen is essentially salted out of saturated aqueous brine solutions (the head group region has a charge density similar to saturated brine) and (2) that the head group region is essentially crystalline, not liquid, and dioxygen tends to be excluded from ionic crystalline materials. However, making this zero head group solubility assumption increases our K'_{par} value by less than 10% over the value obtained by applying the Subczynski and Hyde assumption to our data above the 24°C phase transition. Below the 24°C phase transition the nature of the head group assumption is more critical. At 20°C, where we determined K_{par} to be 1.2, we calculate $K'_{par} = 1.8$ using our zero head group solubility assumption and $K'_{par} = 1.3$ using the Subczynski and Hyde assumption.

The aqueous solubility of dioxygen in air saturated fresh water at 25°C is 0.25 mM while the value in liquid hydrocarbons (and most other simple organic solvents) is about 2 mM. Thus if the hydrocarbon chains of the bilayer resembled an isotropic solvent one might expect the K'_{par} value at this temperature to be about 2/0.25 = 8. De Young and Dill [19] used hexane partitioning data to study how K'_{par} is reduced on going from a isotropic hydrocarbon solvent into the anisotropic hydrocarbon chains of a bilayer membrane. They propose that the concentration of a nonpolar solute will approach the isotropic solvent value only at mid-bilayer. These workers predict a solute concentration profile across the bilayer similar to that found experimentally by White [20] using neutron diffraction on pd-hexane in a bilayer membrane or the one for dioxygen in a bilayer membrane suggested by EPR spin label results [17,8]. Our value of K'_{par} for dioxygen in DMPC bilayers at 25°C of about 5 seems reasonable in view of this theory.

Acknowledgements

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References

1 Power, Gordon G. and Stegall, H. (1970) J. Appl. Physiol. 29, 145–149.
2 Fischkoff, S. and Vanderkooi, J. (1975) J. Gen. Physiol. 65, 663–676.
3 Vanderkooi, J.M., Wright, W.W. and Erecinska, M., D.F. (1990) Biochem. 29, 5332–5338.
4 Kimmich, R., Peters, A. and Spohn, K.H. (1981) J. Membr. Sci. 9, 313–336.
5 Subczynski, W.K. and Hyde, J.S. (1983) Biophys. J. 41, 283–286.
6 Hyde, J.S. and Subczynski, W.K. (1988) Spin Label Oximetry. In Spin Labeling IV: Theory and Applications (Berliner, L.J., ed.), Academic Press, New York.
7 Swartz, H. and Glockner, J. (1989) Measurements of the Concentration of Oxygen in Biological Systems Using EPR Techniques. In Advanced EPR in Biology and Biochemistry, (Hoff, A.J., ed.), pp. 753–782, Elsevier, Amsterdam.
8 Subczynski, W.K., Hyde, J.S. and Kusumi, A. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 4474–4478.

9 Standard Methods for the Examination of Wastewater (1971) 13th edn., American Public Health Association Inc.
10 Hitchman, Michael L. (1978) in Measurement of Dissolved Oxygen, John Wiley & Sons, New York.
11 Willard, H.H., Merritt, L.L. and Dean, J.A. (1974) Instrumental Methods of Analysis, D. Van Nostrand, New York.
12 Nagle, J.F. and Wilkinson, D.A. (1978) Biophys. J. 23, 159–175.
13 Katz, Y. and Diamond (1974) J. Memb. Biol. 17, 87–100.
14 Jamieson, D.D. (1989) Free Radical Biol. Med. 7, 87–108.
15 Imlay, J.A. and Linn, S. (1988) Science 240, 1301–1309.
16 Friedovich, F. (1989) J. Biol. Chem. 264, 7761–7764.
17 Windrem. D. and Plachy, W. (1980) Biochim. Biophys. Acta. 600, 655–655.
18 Battino, R., Ed. (1981) Oxygen and Ozone, (Solubility Data Series IUPAC), Vol. 7, Pergamon Press, Oxford.
19 De Young, L.R. and Dill, K.A. (1990) J. Phys. Chem. 94, 801–809.
20 White, S., King, G.I. and Cain, J.E. (1981) Nature 290, 161–163.