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STUDIES ON THE "FACILITATED FLUX" OF OXYGEN THROUGH HEMOGLOBIN SOLUTIONS USING A POLAROGRAPHIC METHOD

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SUMMARY

The phenomenon of hemoglobin-facilitated O_2 diffusion was studied by a polarographic method.

Polarograms relative to the reduction process of O_2 have been obtained at pH 7.2 (phosphate buffer, 30°) in the presence of various hemoglobin concentrations (Hb_{tot}*) and at various O_2 partial pressures (from 8 to 360 mm Hg).

Analogous experiments were performed at pH 6.4 and 8.1 (at constant ionic strength). Graphs of the limiting current values (at $E = -1.5$ V versus the saturated calomel electrode), relative to the overall reduction process of oxygen, plotted *versus* P_{O_2} (at Hb_{tot}* = constant), show some characteristic trends. The influence of pH on the features of the experimental curves is discussed.

Experimental results suggest that the diffusions of O_2, oxyhemoglobin and hemoglobin, as well as the kinetics of dissociation and association of O_2 with hemoglobin, are effective in determining the "facilitated flux".

The corresponding nonlinear differential system is solved under some simplifying assumptions, and an expression for the flux, and consequently for the current, is obtained which is consistent with the experimental findings.

Furthermore, it is shown that the dissociation curve of oxyhemoglobin can be obtained from these polarographic experiments on the basis of this theory. Agreement with tensiometric data was satisfactory.

INTRODUCTION

The phenomenon of the "facilitated flux" of O_2 in hemoglobin solutions has been the subject of a large number of investigations with tensiometric methods[1-3]; some experiments have been carried out in order to clarify the phenomenon[4-10] and various theoretical interpretations have been proposed[11-16].

We have tried to make a contribution to the study of the phenomenon by using a polarographic method. This method offers, among other things, two noticeable advantages: first, it is easy to perform the experiments over a wide range of O_2 pressures (starting from P_{O_2} of approx. 10 mm Hg); furthermore, the operative conditions of the polarographic method are such that, at the solution–electrode interface, the O_2 concentration is actually zero when the limiting current of the overall reduction process is reached.

EXPERIMENTAL

The experiments were performed with pig blood. Defibrinated blood was washed 3 times with physiological saline (0.9 % NaCl, pH 7), hemolyzed and centrifuged. The percentage of hemoglobin was evaluated with an Erke hemometer using doubled colored sheets.

As the percentage of hemoglobin is not always the same in different blood samples, the solutions were suitably diluted with phosphate buffer (pH 7.2) so that every sample contained 8 % hemoglobin. These samples, stored at $-4°$, were diluted with buffer just before the measurements to give the following hemoglobin concentrations: 0.8, 1.6, 2.4, 3.2, 4.0 and 4.8 %.

Ferric hemoglobin solutions were prepared by the conventional technique from ferrohemoglobin solution and $K_3Fe(CN)_6$ in stoichiometric ratio and then dialyzed again.

The capillary used had a drop time of 2.8 sec at the applied potential of -1.5 V and $h = 1$ m (h = mercury reservoir height).

O_2 solutions were prepared by saturating the solvent system with the following gaseous O_2–N_2 mixtures: 1.05, 1.9, 2.6, 4, 5.05, 8, 12, 15, 30, 40, 50 and 100 %.

The solutions, when necessary, were de-aerated by bubbling through very pure N_2. Particular care was taken to reach the solution equilibrium of O_2 at the various partial pressures by recording the limiting current of O_2 as a function of the time until a constant value was attained.

Minute quantities of octylic alcohol were added to the test solutions to avoid foaming as a consequence of the gas bubbling; this had no effect on the height and shape of the diffusion current of O_2.

Throughout the experiments Merck or Carlo Erba highly pure pro analysis reagents were used.

The diffusion currents were corrected for the base current; all potentials are referred to the 'saturated calomel electrode'. Polarographic measurements were carried out using a Model XV Sargent polarograph. pH was measured using an Amel 331 pH meter.

RESULTS

We have recorded polarograms of the reduction process of O_2 at pH 7.2 (temp. 30°) in the presence of various hemoglobin concentrations (0.8, 1.6, 2.4, 3.2, 4 and 4.8 %) and at various O_2 partial pressures; the results are shown in Figs. 1a and 1b. Here the limiting diffusion current of oxygen *versus* O_2 partial pressures from 8 to 115 mm Hg are indicated. The potential is $E = -1.5$ V, which is relative to the overall reduction process of O_2 ($O_2 + 2H_2O + 4e \rightleftharpoons 4OH^-$). In Fig. 2 the diffusion limiting currents, obtained in the absence of hemoglobin (*i.e.* in phosphate buffer, pH 7.2) or in the presence of ferric hemoglobin (which does not bind O_2), are reported. In this last instance, it can be noted that the presence of ferric hemoglobin does not modify the linear behavior of the diffusion current as a function of P_{O_2}, showing that the diffusion coefficient of O_2 is practically unchanged, at least with the low concentrations of hemoglobin used.

The curves of Fig. 1 show the following peculiarities: (a) Starting from a value

for the O_2 partial pressure of about 40 mm Hg (the same for all hemoglobin concentrations), the limiting diffusion current varies linearly with the P_{O_2} (hence with the concentration of O_2 dissolved in the solution). For P_{O_2} values smaller than 40 mm Hg, we note a progressive bending, the more remarkable the higher the concentration of

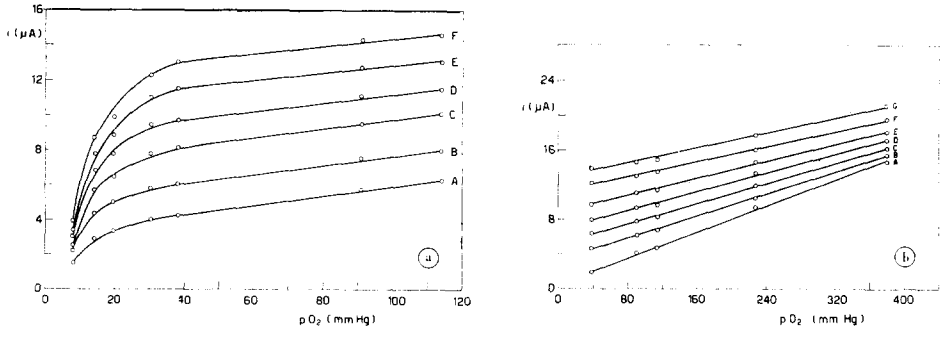

Fig. 1. Limiting current values for O_2 reduction, as a function of P_{O_2}, in Hb solutions. Phosphate buffer (pH 7.2); 30°. (a) P_{O_2} ranging from 8 up to approx. 115 mm Hg. (b) P_{O_2} ranging from 40 to approx. 380 mm Hg. Hb_{tot}* concentrations: A, 0.8%; B, 1.6%; C, 2.4%; D, 3.2%; E, 4.0%; F, 4.8%. Polarographic conditions as indicated in the text.

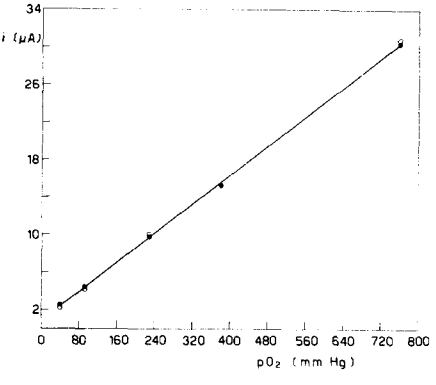

Fig. 2. Limiting current values for O_2 reduction, as a function of P_{O_2}, in phosphate buffer (pH 7.2); 30°. ○, without ferric hemoglobin; ●, with 0.8% ferric hemoglobin. Polarographic conditions as indicated in the text.

hemoglobin in the solution; this behavior is clearly connected with the dissociation curve of oxyhemoglobin. (b) The slope of the linear portion of the curves, corresponding to various hemoglobin concentrations, is lower than in the absence of hemoglobin (*i.e.* in the buffer alone) (Figs. 1 and 2). From these curves, the phenomenon of facilitated flux of O_2 in the presence of a hemoglobin solution is clearly shown. For example, the limiting current values (at 40 mm Hg) increase from 2 to 13.3 μA when hemoglobin concentrations are zero and 4.8%, respectively.

Electrocapillary curves of 0.8 and 3.2% have shown that hemoglobin is strongly adsorbed near the electrocapillary maximum, but at the potential of -1.5 V, where diffusion currents were recorded, desorption is practically complete.

Experiments like that summarized in Fig. 1 (pH 7.2) have also been performed at pH 6.4 and 8.1 (at constant ionic strength $I = 0.82$ in phosphate buffer) with anal-

ogous results; the graphs are given in Figs. 3a and 3b for a hemoglobin concentration of 0.8 %.

The most interesting result of these measurements is that the linear trend of the current as a function of P_{O_2}, which indicates saturation of hemoglobin with O_2 in the solution, begins at $P_{O_2} = 45$ mm Hg for pH 6.4, at $P_{O_2} = 40$ mm Hg for pH 7.2, whereas for pH 8.1 it is already attained at the lowest P_{O_2} (8 mm Hg) used.

Another feature is that the slope of the straight lines, corresponding to saturation, is slightly higher at lower pH, *i.e.* in connection with higher $P_{O_2}½$ (the O_2 partial pressure value at 50 % saturation). Furthermore, the value of the current, extrapolated at $P_{O_2} = 0$ (a value which, as we shall see, can be assumed as a measure of the facilitated flux), increases as pH decreases.

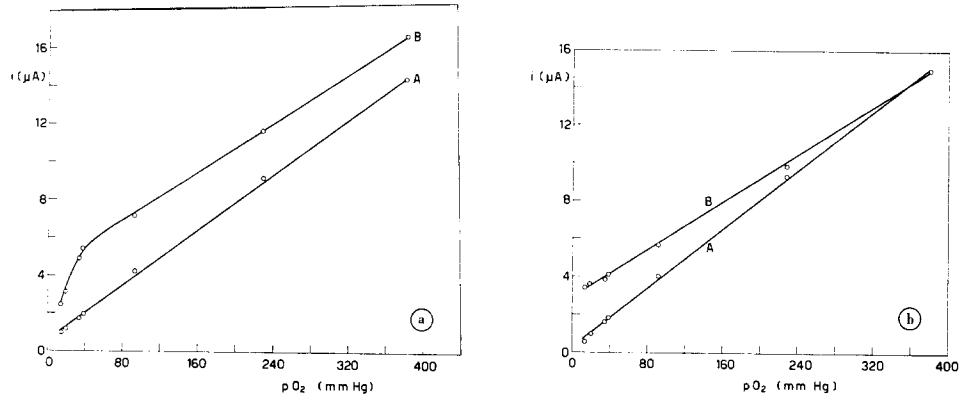

Fig. 3. Limiting current values for O_2 reduction, as a function of P_{O_2}, in phosphate buffer; 30°. (a) pH 6.4: A, $c_{Hb,tot}^* = 0$; B, $c_{Hb,tot}^* = 0.8\%$. (b) pH 8.1: A, $c_{Hb,tot}^* = 0$; B, $c_{Hb,tot}^* = 0.8\%$. Polarographic conditions as indicated in the text.

DISCUSSION

The processes with which the O_2 is concerned under our experimental conditions are:

(i) Electrochemical reduction, which at the potential $E = -1.5$ V of the limiting diffusion current can be written:

$$O_2 + 4e + 2H_2O \rightleftharpoons 4OH^- \qquad (1)$$

(ii) The chemical reaction of O_2 with hemoglobin (Hb):

$$HbO_2 \rightleftharpoons Hb + O_2 \qquad (2)$$

exhibiting a rate:

$$v = kc_{HbO_2} - k'c_{Hb}c_{O_2} \qquad (3)$$

where c_{HbO_2}, c_{Hb}, c_{O_2} represent the actual concentrations of oxyhemoglobin, hemoglobin and O_2, respectively, at a distance x from the electrode and at time t, k and k' being the rate constants of dissociation and association, respectively.

(iii) Diffusion, described by Fick's second law and modified by the chemical reaction with a rate expressed by Eqn. 3.

At the same time, diffusion also concerns oxyhemoglobin and hemoglobin which are present in the solution, and Fick's second law, with the proper boundary conditions, applies to such substances which, like O_2, are involved in Reaction 2 (*cf.* APPENDIX).

The solution of the differential problem, in its more general formulation (*i.e.* in the case in which the diffusion coefficients of $O_2(D_{O_2})$ and of hemoglobin (D_{Hb}) are considered to be different), presents noticeable difficulties, unless one resorts to numerical methods, because of the kinetic term which is nonlinear with respect to the concentrations. For investigating the facilitated flux and for obtaining a semiquantitative interpretation of its nature, we have found useful an approximation by which a diffusion coefficient D_{O_2} of oxygen $\gg D_{Hb}$ of hemoglobin can be assumed (in fact, $D_{O_2} \approx 300\ D_{Hb}$ (ref. 18)). Furthermore, since at the electrode ($x = 0$) the O_2 concentration $c_{O_2}(0,t) = 0$ when a diffusion limiting current is concerned, $c_{HbO_2}(x,t) < c_{Hb,tot}^*$ for x values near the electrode, ($c_{Hb,tot}^* = c_{HbO_2}^* + c_{Hb}^* = c_{HbO_2}(x,t) + c_{Hb}(x,t)$, if $D_{HbO_2} = D_{Hb}$).

With such an approximation, we obtain for the current (*cf.* APPENDIX):

$$i = at^{1/6}\sqrt{\bar{D}}[c_{O_2}^* + c_{HbO_2}^* f(\sqrt{kt})] \tag{6}$$

where a is a constant depending on the characteristics of the capillary,

$$\bar{D} = \frac{D_{O_2}}{1 + \frac{k'}{k} c_{Hb,tot}^*} \tag{7}$$

and $f(\sqrt{kt})$ is a function of the dissociation rate constant k of oxyhemoglobin. Taking into account that $c_{HbO_2} = y(P_{O_2})c_{Hb,tot}^*$, where $y(P_{O_2})$ represents the dissociation curve of oxyhemoglobin, it can be noted that $y \to 1$ for P_{O_2} values near saturation and hence

$$i = at^{1/6}\sqrt{\bar{D}}[c_{O_2}^* + c_{Hb,tot}^* f(\sqrt{kt})] \tag{8}$$

The total polarographic current therefore appears to be the sum of two contributions. The former comes from the O_2 physically dissolved in the solution (first addendum in Eqn. 6 or 8), and the second comes from the hemoglobin-bound O_2 (the second addendum in Eqn. 6 or 8).

Eqn. 8 predicts that, for a fixed Hb_{tot}^* concentration, the current will vary linearly with the concentration of O_2 dissolved in the solution (hence with P_{O_2}), when saturation is nearly achieved.

The proportionality coefficient between the current and P_{O_2} depends in turn on $\bar{D}$, where $\bar{D}$ is the quantity defined by Eqn. 7.

From Eqn. 6 we obtain:

$$y(P_{O_2}) = \frac{i - at^{1/6}\sqrt{\bar{D}}c_{O_2}^*}{at^{1/6}\sqrt{\bar{D}}c_{Hb,tot}^* f(\sqrt{kt})} \tag{9}$$

i.e. the saturation curve of hemoglobin.

The graphs drawn from Fig. 1 according to Eqn. 9 are shown in Fig. 4. As predicted by Eqn. 9, the curves of Fig. 4 are almost independent of the concentration of hemoglobin.

To test the reliability of our formulation and the limits of our hypotheses, we have obtained from Fig. 4 the values of n and $P_{O_2,½}$ of Hill's equation (see for example ref. 19): $n = 2.8$ and $\log P_{O_2,½} = 1.05$ (phosphate buffer (pH 7.2)). Tensiometric measurements gave $n = 2.8$; $\log P_{O_2,½} = 0.98$ at 30°, (phosphate buffer (pH 7.2)) for the same hemoglobin sample used in the polarographic experiments. The good agreement between polarographic and tensiometric data support the likelihood of the proposed mechanism.

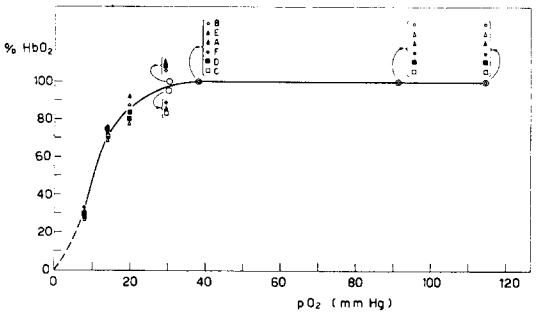

Fig. 4. Dissociation curve of HbO_2 according to Eqn. 9 of the text and data of Fig. 1a.

The value of the current extrapolated at $c_{O_2} = 0$ in Eqn. 8 thus gives a measure of the facilitated flux. For a given concentration of hemoglobin Hb_{tot}*, this quantity depends on the value of the function $f(\sqrt{kt})$. This is equal to zero for $kt = 0$, approaches 1 for $kt \to \infty$, and is an increasing function of kt; for a fixed value of t, it is therefore an increasing function of k.

The data of Fig. 3 can also be interpreted in the same way.

It is known[9,20] that the dissociation rate constant k diminishes as pH increases, hence the second term of Eqn. 8 which is proportional to the facilitated flux also decreases with increases in pH.

The change of $P_{O_2,½}$ with the pH ($\log P_{O_2,½}$ diminishes as pH increases) also conforms to the "Bohr effect" (ref. 19).

It is well known that the kinetics of the reaction of hemoglobin with ligands can be accounted for by Eqn. 3 only as a first approximation. On the other hand, the simplifying hypotheses we have introduced can be justified by the agreement between the experimental results and the consequences foreseen on the basis of such hypotheses.

APPENDIX

The concentrations of O_2, HbO_2 and Hb, in the most general case, must conform to the equations (in the planar linear approximation):

$$\frac{\delta c_{O_2}}{\delta t} = D_{O_2} \frac{\delta^2 c_{O_2}}{\delta x^2} + k c_{HbO_2} - k' c_{Hb} c_{O_2} \qquad \text{(A1)}$$

$$t = 0, \qquad x \geqslant 0, \qquad c_{O_2} = c_{O_2}^* \qquad \text{(A2)}$$

$$t > 0, \qquad x = 0, \qquad c_{O_2} = 0 \qquad \text{(A3)}$$

$$\lim_{x \to \infty} c_{O_2} = c_{O_2}^* \tag{A4}$$

$$\frac{\delta c_{HbO_2}}{\delta t} = D_{Hb} \frac{\delta^2 c_{HbO_2}}{\delta x^2} - k c_{HbO_2} + k' c_{Hb} c_{O_2} \tag{A5}$$

$$t = 0, \qquad x \geqslant 0, \qquad C_{HbO_2} = c_{HbO_2}^* \tag{A6}$$

$$t > 0, \qquad x = 0, \qquad D_{Hb} \left(\frac{\delta c_{HbO_2}}{\delta x} \right)_{x=0} = 0 \tag{A7}$$

$$\lim_{x \to \infty} c_{HbO_2} = c_{HbO_2}^* \tag{A8}$$

$$\frac{\delta c_{Hb}}{\delta t} = D_{Hb} \frac{\delta^2 c_{Hb}}{\delta x^2} + k c_{HbO_2} - k' c_{Hb} c_{O_2} \tag{A9}$$

$$t = 0, \qquad x \geqslant 0, \qquad c_{Hb} = c_{Hb}^* \tag{A10}$$

$$t > 0, \qquad x = 0, \qquad D_{Hb} \left(\frac{\delta c_{Hb}}{\delta x} \right)_{x=0} = 0 \tag{A11}$$

$$\lim_{x \to \infty} c_{Hb} = c_{Hb}^* \tag{A12}$$

where $c_{O_2}^*$ and c_{HbO_2} represent the concentration of O_2 and oxyhemoglobin in equilibrium with each other in the bulk of the solution.

Eqn. A3 expresses the condition for the diffusion limiting current; Eqns. A7 and A11 mean that neither HbO_2 nor Hb are reduced at the electrode. Any adsorption or kinetic complications different from that described by Eqn. 2 of the text are excluded. (We have assumed $D_{HbO_2} = D_{Hb}$.)

From Eqns. A5–A12 it can easily be verified that

$$c_{HbO_2}(x,t) + c_{Hb}(x,t) = c_{HbO_2}^* + c_{Hb}^* = c_{Hb,tot}^* \tag{A13}$$

The solution of Eqns. A1–A12 offers noticeable difficulties because of the presence of the kinetic term which is nonlinear with respect to the concentrations.

From Eqns. A1 and A13 we obtain

$$\frac{\delta c_{O_2}}{\delta t} = D_{O2} \frac{\delta^2 c_{O_2}}{\delta x^2} + k c_{HbO_2} - k'(c_{Hb,tot}^* - c_{HbO_2}) c_{O_2} \tag{A14}$$

Assuming that $c_{HbO_2}(x,t) \ll c_{Hb,tot}^*$ for x values near the electrode, Eqn. A14 reduces to

$$\frac{\delta c_{O_2}}{\delta t} = D_{O_2} \frac{\delta^2 c_{O_2}}{\delta x^2} + k c_{HbO_2} - k' c_{Hb,tot}^* c_{O_2} \tag{A15}$$

Actually, $D_{Hb} \ll D_{O_2}$ (D_{Hb}/D_{O_2} approximates 0.03), hence

$$\frac{\delta c_{HbO_2}}{\delta t} = - k c_{HbO_2} + k' c_{Hb,tot}^* c_{O_2} \tag{A16}$$

Let $L\{c_{HbO_2}(x,t)\} = u(x,P)$

and $L\{c_{O_2}(x,t)\} = v(x,P)$

be the Laplace transformations of $c_{HbO_2}(x,t)$, $c_{O_2}(x,t)$ with respect to time t. By transforming Eqns. A15 and A16 we obtain

$$Pv - c_{O_2}^* = D_{O_2} v''_{xx} + ku - k' c_{Hb,tot}^* v \tag{A17}$$

$$Pu - c_{HbO_2}^* = -ku + k' c_{Hb,tot}^* v \tag{A18}$$

It follows that

$$\frac{P}{D_{O_2}}\left(1 + \frac{k'}{P+k} c_{Hb,tot}^*\right) v = v''_{xx} + \frac{1}{D_{O_2}}\left(c_{O_2}^* + \frac{k}{P+k} c_{HbO_2}^*\right) \tag{A19}$$

Under the conditions stated, Eqn. A19 can be solved for $v(x,p)$. Since we are mainly interested in the flux $D_{O_2}(\delta c_{O_2}/\delta x)_{x=0}$ we obtain $\varphi(0,P) = L\{D_{O_2}(\delta c_{O_2}/\delta x)_{x=0}\} = D_{O_2}(\delta v/\delta x)_{x=0}$ which is given by

$$\varphi(0,P) = \frac{\sqrt{D_{O2}}\left(c_{O_2}^* + \frac{k}{P+k} c_{HbO_2}^*\right)}{\sqrt{1 + \frac{k'}{k} c_{Hb,tot}^* \frac{1}{\frac{P}{k}+1}}} \cdot \frac{1}{\sqrt{P}} \tag{A20}$$

The inverse transformation of (A20) can easily be performed in the case $P/k \gg 1$ or $P/k \ll 1$. The last figure corresponds to $t \gg 1/k$; since k is on the order of 10 sec^{-1}, and $t > 1$ sec, as far as our polarographic measurements are concerned, it does not seem inaccurate to represent Eqn. A20 as

$$\varphi(0,P) = \frac{\sqrt{D}_{O_2}}{\sqrt{1 + \frac{k'}{k} c_{Hb,tot}^*}} \cdot \frac{c_{O_2}^*}{\sqrt{P}} + \frac{\sqrt{D}_{O_2} c_{HbO_2}^*}{\sqrt{1 + \frac{k'}{k} c_{Hb,tot}^*}} \frac{k}{\sqrt{P}(P+k)} \tag{A21}$$

The inverse transformation of Eqn. A21 is

$$D_{O_2}\left(\frac{\delta c_{O_2}}{\delta x}\right)_{x=0} = \sqrt{D}\frac{c_{O_2}^*}{\sqrt{\pi t}} + \sqrt{D}\frac{c_{HbO_2}^*}{\sqrt{\pi t}} f(\sqrt{kt}) \tag{A22}$$

where

$$D = \frac{D_{O_2}}{1 + \frac{k'}{k} c_{Hb,tot}^*} \tag{A23}$$

and

$$f(\sqrt{kt}) = \sqrt{\pi}\ \sqrt{kt} e^{-kt} \frac{2}{\sqrt{\pi}} \int_0^{\sqrt{kt}} e^{\lambda^2} d\lambda \tag{A24}$$

It follows that the current $i = at^{2/3} D_{O_2} (\delta c_{O_2}/\delta x)_{x=0}$ is given by

$$i = at^{1/6}\sqrt{D}[c_{O_2}^* + c_{HbO_2}^* f(\sqrt{kt})] \tag{A25}$$

i.e. Eqn. 6 of the text.

The function

$$f(\sqrt{kt}) = \sqrt{\pi}\sqrt{kt}\, e^{-kt}\frac{2}{\sqrt{\pi}}\int_0^{\sqrt{kt}} e^{\lambda^2} d\lambda \qquad (A26)$$

ranges between 0 and 1.

For $kt \to \infty$ (*i.e.* for very high values of the dissociation constant), Eqn. A25 reduces to

$$i = at^{1/6}\sqrt{D}(c_{O_2}{}^* + c_{HbO_2}{}^*) \qquad (A27)$$

i.e. the current should be proportional to the overall O_2 bulk concentration, free and hemoglobin bound.

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