

Luminescence Ratio Indicators for Oxygen

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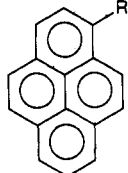
To improve stability of optical sensors with respect to calibration, we have developed oxygen indicator systems which have two luminescence bands, one of which is quenched by oxygen while the other is relatively unaffected by oxygen. The measured parameter is the relative intensity of the two bands. The following systems have been evaluated: (1) In water in the presence of γ -cyclodextrin, the ratio of pyrene monomer fluorescence to pyrene dimer fluorescence decreases with increasing oxygen partial pressure. (2) In water in the presence of β -cyclodextrin, the ratio of bromonaphthalene phosphorescence to fluorescence decreases with increasing oxygen partial pressure. (3) Bromonaphthoyl derivatives extracted into γ -cyclodextrin bonded to cellulose and dried show intense oxygen-dependent phosphorescence and oxygen-independent fluorescence. Of these, system 3 is most practical because it is more stable and has higher intensities with a larger change in intensity ratio. However, it is limited to measurements in dry samples, because 100% relative humidity is sufficient to quench phosphorescence.

Considerable effort has been devoted to the development of an optical sensor that responds to oxygen on an equilibrium basis. Fluorescence quenching has been the preferred approach to date (1-8) although the possibility of basing a device on spectral shifts accompanying reversible oxygenation has also been explored (9). Unlike the amperometric oxygen electrode, the optical device does not require a steady-state supply of oxygen to the sensor surface for constant response.

To maximize stability with respect to calibration, it is desirable to design optical sensors so that the magnitude of the analytical intensity can be measured relative to a reference intensity. The ideal reference intensity would compensate not just for instrumental fluctuations but also for variations in optical properties of the sample and slow loss of reagent due to degradation or leaching. In optical oxygen sensors reported to date, reference intensities have been based on the measurement of either scattered excitation radiation (4) or emission from a second oxygen-insensitive fluorophor included in the reagent phase of the sensor (6).

The goal of the work reported here was to develop a single reagent that would show two luminescence bands, an "analytical" band subject to quenching by oxygen and a "reference" band independent of oxygen levels. Two approaches were explored. One approach is to design the system so that a fluorescent reagent is in equilibrium between two forms with different emission bands, one of which is oxygen sensitive. Only pyrene and its derivatives were considered because they have longer fluorescence lifetimes than most other fluorophors, allowing more time for significant oxygen quenching. The specific goal was to formulate a system that

Table I. Ratio of Fluorescence Intensities in Argon- vs. Air-Saturated Water for Ionic Pyrene Derivatives

	R	name	I_{Ar}/I_{O_2}
	$-\text{CH}_2\text{N}(\text{CH}_3)_3^+$	PMTA ⁺	2.7
	$-(\text{CH}_2)_3\text{COO}^-$	PBA	2.6
	$-\text{COO}^-$		1.7
	$-\text{SO}_3^-$		1.7
	$-\text{CH}=\text{CHCOO}^-$		1.0

would show both oxygen-sensitive pyrene monomer emission and oxygen-insensitive pyrene dimer emission at longer wavelength.

The second approach is to find lumophores that show both oxygen-insensitive fluorescence and oxygen-sensitive phosphorescence. Because phosphorescence from fluid solutions is usually completely quenched, the problem here is to choose a system that shows measurable phosphorescence in the presence of oxygen. It has been demonstrated that bromonaphthalene and some of its derivatives form inclusion complexes with cyclodextrins that protect the lumophor from oxygen quenching, allowing it to phosphoresce in fluid solution in the presence of oxygen (10-13). Because bromonaphthalene and its derivatives also fluoresce, they were the system of choice in developing a two-wavelength oxygen indicator.

EXPERIMENTAL SECTION

Apparatus. The effect of oxygen on luminescence was measured with a fiber-optic filter fluorometer, including a Model 6254 150-W xenon source from Oriol Corp., a $1/4$ -in.-diameter bifurcated bundle of 0.010-in.-diameter quartz fibers from Dolan Jenner, mechanically actuated filter wheels for selection of both excitation and emission wavelengths, and a Hamamatsu R763 photomultiplier tube detector operated at 800 V. Signals were read out on an Allen datagraph strip chart 2125B strip chart recorder and a Fluke 8050A digital multimeter. Either a special gas chamber or the sample compartment of an Instrumentation Laboratory 1301 blood gas analyzer was used as the sample chamber.

Gas mixtures were prepared by using a PGM-3 precision gas mixer.

Luminescence spectra were measured on either Perkin-Elmer MPF 44B or MPF 2A spectrofluorometers.

Materials. Nitrogen (ultrahigh purity) and oxygen (extra dry) were obtained from Air Products and Chemicals, Inc. β - and γ -cyclodextrin covalently bound to cellulose resins were obtained from Advanced Separation Technologies. Except for pyrenebutyric acid (PBA), which was obtained from Aldrich, all the ionic pyrene derivatives listed in Table I were obtained from Molecular Probes, Inc. Bromonaphthoyl derivatives were prepared by literature methods (14). In brief, the procedure involves reacting 1-bromonaphthalene with the acid chloride of a bromine-substituted carboxylic acid. The bromine is then reacted with excess trimethylamine to form the quaternary ammonium salt (5-(4-bromo-1-naphthoyl)pentyltrimethylammonium bromide (BNK5⁺) and 10-(4-bromo-1-naphthoyl)decyltrimethylammonium bromide

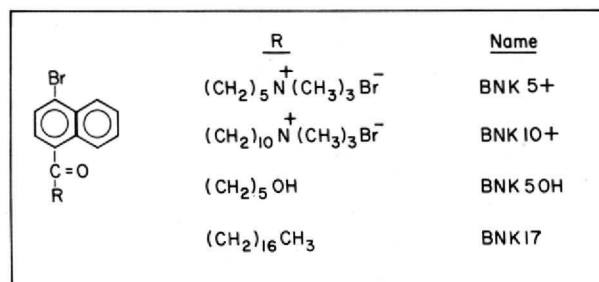


Figure 1. 4-Bromo-1-naphthyl derivatives used in this study.

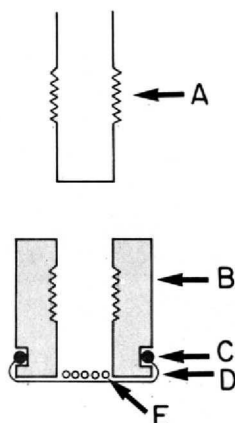


Figure 2. Diagram of sensor. A is the common end of a bifurcated fiber-optic bundle. (B) is a cross section of the sensor cap which screws onto A. C is an O-ring which holds oxygen-permeable membrane D over the end of the sensor cap. E is the oxygen-sensitive reagent.

(BNK10⁺) or hydrolyzed (5-(4-bromo-1-naphthoyl)-1-pentanol (BNK5OH)). (BNK17) was prepared by reacting octadecanoyl chloride with 1-bromonaphthalene. Structures were confirmed by ¹³C NMR. The spectra were clean, showing no trace of impurities. Figure 1 shows structures and nomenclature for the bromonaphthoyl compounds used in this study. All other reagents were obtained from Aldrich.

Procedures. Inclusion complexes in β - and γ -cyclodextrins immobilized on cellulose were prepared by mixing approximately 50 mg of solid resin with 10 mL of a 10 mM solution of the lumophore in an appropriate solvent. After several hours was allowed for equilibration, the solid phase was separated by filtration and allowed to air-dry. The solvents were water for BNK5⁺ and BNK10⁺, tetrahydrofuran for BNK5OH, and diethyl ether for BNK17. Some preparations of BNK5⁺ on γ -CD resin included 0.10 M of a salt with a hydrophobic anion that would displace the bromide in the solid-phase complex.

Luminescence spectra of solid materials were measured at 45° by using double-sided cellophane tape. One side of the tape was stuck to a microscope slide while the other was contacted with the solid of interest.

The oxygen sensor arrangement is shown in Figure 2. A small amount (ca. 2 mg) of solid-phase indicator was placed in the sensor cap, which was then screwed onto the common end of the bifurcated fiber-optic bundle. The oxygen-permeable membrane over the end of the sensor cap was pressed firmly onto the solid material to prevent it from moving and to minimize the dead volume of oxygen in the indicator area. The oxygen-permeable membrane was either porous polypropylene or porous Teflon (Gore-tex).

RESULTS AND DISCUSSION

Pyrene Monomer-Dimer System. γ -Cyclodextrin (γ -CD) was added to aqueous pyrene solutions to induce ground-state pyrene dimer formation. Figure 3 shows the luminescence emission spectrum from 0.50 μ M pyrene in the presence of 10 mM γ -CD. Clearly resolved pyrene monomer and dimer bands are observed at 360–420 nm and 440–560 nm, respectively. Using literature equilibrium constant values for the

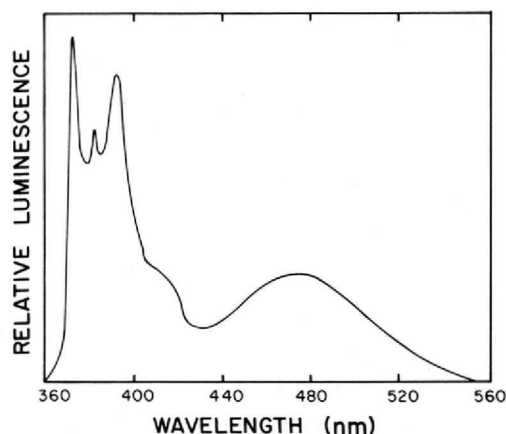


Figure 3. Monomer and dimer fluorescence from 0.50 μ M pyrene in the presence of 10 mM γ -CD.

interaction of γ -CD and pyrene (15, 16), we calculated that the above solution contains 0.085 μ M of free pyrene monomer, 0.12 μ M of pyrene monomer bound to CD, and 0.15 μ M of pyrene dimer bound to CD at equilibrium. The ratios of intensities in oxygen-saturated solution to intensities in argon-saturated solution are 2.3 and 1.1 for the monomer and dimer bands, respectively. This is consistent with literature lifetime values of 250 and 77 ns for the monomer and dimer bands, respectively (17).

Although the above system exhibits the necessary requirements for two-wavelength oxygen sensing, it has several drawbacks. First, it would be more desirable to be able to excite at longer wavelengths where incandescent sources are more intense and glass optical fibers have higher transmittances. Second, the relative change in the intensity of the "analytical" band is small. Third, a slow precipitation process appears to occur over several days, removing γ -CD/pyrene from solution. (Indeed, slow precipitation of cyclodextrin inclusion complexes was a problem throughout this study. Filtered solutions that appeared to be perfectly clear gave non-reproducible spectra that were subsequently shown to be due to fine particles.) Fourth, because the equilibrium ratio of monomer to dimer varies with total pyrene concentration, the use of dimer emission as a reference band would not fully compensate for slow pyrene degradation.

A variety of approaches for improving the characteristics of the pyrene monomer-dimer system were considered. Ionic pyrene derivatives are much more soluble in water than pyrene. However, as shown in Table I, ionic pyrene has reduced susceptibility to oxygen quenching relative to pyrene. Only PBA and PMTMA⁺ have values large enough for a sensor. PBA in the presence of γ -CD shows monomer and dimer emission bands. However, the ratio of intensities in oxygen- vs. argon-saturated solutions is 1.9 for the monomer band and 1.3 for the dimer band. This difference is insufficient for a sensor.

One approach that was considered but not attempted is to use solvent systems other than water. While it is likely that this approach could lead to systems with improved luminescence response to oxygen, it would lead to a Pandora's box of new problems if such an indicator system were to be applied to the analysis of oxygen in aqueous samples.

Bromonaphthalene. Turro and co-workers have shown that β -cyclodextrin (β -CD) and 1-bromonaphthalene (1-BrNa) form an inclusion complex which exhibits measurable phosphorescence in aerated solution (10). Addition of up to 10% acetonitrile was found to enhance phosphorescence intensity.

We have observed even greater enhancements when either isobutyl alcohol or isoamyl alcohol are substituted for the acetonitrile. Figure 4 shows the luminescence emission spectra

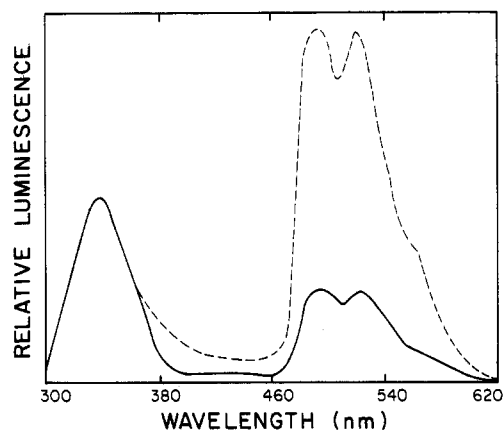


Figure 4. Luminescence spectra of 1-BrNa in the presence of 10 mM β -CD and 5% isoamyl alcohol in air- (—) and nitrogen- (---) saturated aqueous solution.

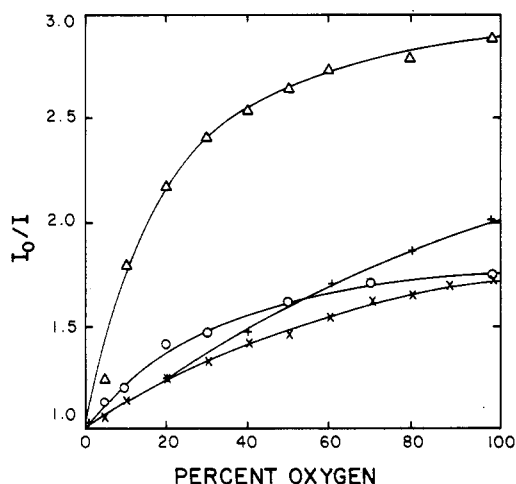


Figure 5. Stern-Volmer plots for 1-BrNa/isoamyl alcohol/ β -CD precipitates particles (X), 1-BrNa/isoamyl alcohol/ β -CD immobilized on cellulose (Δ), BNK5⁺/ γ -CD immobilized on cellulose (+), and BNK17/ γ -CD immobilized on cellulose (O).

of 1-BrNa in air- and nitrogen-saturated solution containing 10 mM of β -CD and 5% isoamyl alcohol. The fluorescence band at 330 nm is unaffected by oxygen, while the phosphorescence band centered at 500 nm is quite sensitive to oxygen. However, the signals from aqueous "solutions" are weak and appear to arise primarily from the surface of fine precipitate particles that form in solution. The effect of deoxygenation on phosphorescence intensity is greatest initially and decreases with time. Furthermore, solutions that are centrifuged do not show significant phosphorescence.

A more practical system results when the precipitate is collected, air-dried, and held on the end of a bifurcated fiber-optic photometer by an oxygen-permeable membrane. Figure 5 shows Stern-Volmer plots for phosphorescence and fluorescence when a precipitate collected from a solution containing 10% isoamyl alcohol was exposed to gas mixtures containing varying percentages of oxygen in the presence of nitrogen. These data exhibit the essential requirements for a luminescence ratio based oxygen indicator. Fluorescence intensities are not affected by oxygen levels at all while phosphorescence changes significantly. The Stern-Volmer plot for phosphorescence is linear up to approximately 40% oxygen. Furthermore, the maximum response time is 30 s and there is no observable hysteresis in the response to increasing vs. decreasing levels of oxygen. As configured, the sensor did not respond to aqueous oxygen samples. However, after the aqueous samples were removed, the reagent continued to respond to oxygen in gas mixtures.

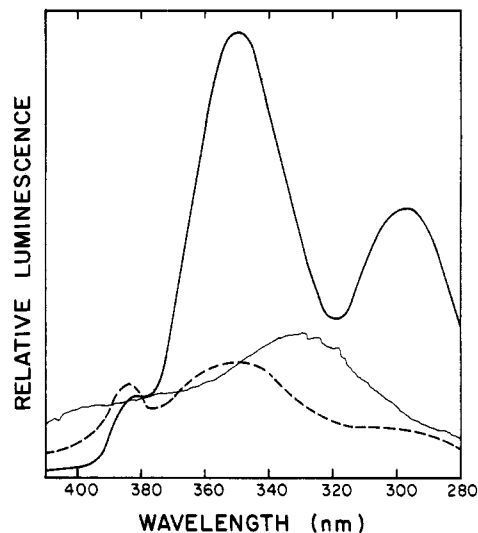


Figure 6. Luminescence excitation spectra (uncorrected) for an aerated aqueous solution of 60 μ M BNK10⁺ including fluorescence excitation in the absence (—) and presence (---) of 10 mM γ -CD, and phosphorescence excitation in the presence of 10 mM γ -CD (—). The emission wavelengths were 440 nm for fluorescence and 550 nm for phosphorescence.

Because 1-BrNa/ β -CD/isoamyl alcohol precipitates could not be prepared reproducibly, a procedure was developed for preparing 1-BrNa inclusion complexes by using β -CD modified cellulose. A suspension of 0.5 g of β -CD modified cellulose, 18 mL of water, 1.0 mL of 1-BrNa, and 1.0 mL of isoamyl alcohol was stirred for 48 h. The product, separated by filtration and air-dried, has spectral characteristics similar to the 1-BrNa/ β -CD/isoamyl alcohol precipitates. As shown in Figure 5, the phosphorescence band is more sensitive to oxygen than for the precipitate while fluorescence remains unaffected by oxygen. The Stern-Volmer plot curves in a manner suggesting that 1-BrNa on β -CD modified cellulose exists in at least two states that differ in their susceptibility to oxygen quenching. Response times to oxygen in gas mixtures were 3–5 min but could be reduced to 30 s or less by grinding the β -CD cellulose to a finer particle size.

While 1-BrNa based indicators meet the requirements for a luminescence ratio based oxygen measurement, they require excitation at 285 nm, well down in the ultraviolet, which in turn significantly increases the cost and complexity of associated optical instrumentation.

Bromonaphthoyl Derivatives. Because of the advantages of excitation at longer wavelength, our attention turned to bromonaphthoyl derivatives first studied by Turro and co-workers (12–14). The absorption and emission spectra for the bromonaphthoyl derivatives used in this study agree with the literature (12, 14). However, luminescence excitation spectra have not been previously reported. They are shown in Figure 6 for an aerated solution of 10-(4-bromo-1-naphthoyl)decyltrimethylammonium bromide (BNK10⁺) in the presence of 10 mM γ -CD. The two fluorescence excitation spectra are on the same scale. However, the gain and bandwidth were both increased to observe the much weaker phosphorescence excitation spectrum. The band at 385 nm in the fluorescence spectra is the water Raman band.

Interestingly, as shown in Figure 6, phosphorescence and fluorescence have different excitation spectra, neither of which match the absorption spectrum which has a maximum at 303 nm (12). Furthermore, addition of γ -CD causes a significant decrease in fluorescence intensity along with changes in the excitation spectrum. These data imply that we are not dealing with a single lumophor. BNK10⁺ (and BNK5⁺) may contain impurities which, although not observed in NMR spectra, luminesce so strongly relative to the BNK compounds that

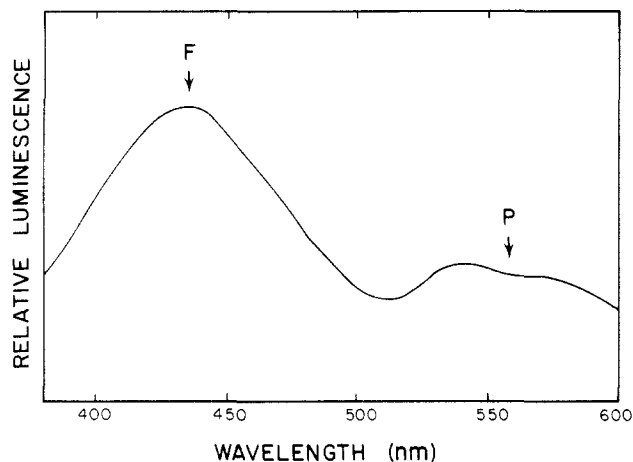


Figure 7. Luminescence emission spectrum (uncorrected) for BNK5⁺ on γ -CD bonded cellulose. Excitation is at 350 nm. F and P indicate the fluorescence and phosphorescence bands, respectively.

they influence the observed excitation spectra. Alternatively, the BNK compounds may exist in more than one form, e.g., dimers with various orientations. In the earlier study with bromonaphthoyl derivatives, phosphorescence lifetimes were biphasic, indicating a two component mixture (12, 14). In any event, it is important to recognize that luminescence behavior of the bromonaphthoyl derivatives is complex and is not completely understood.

Although solutions of BNK10⁺ and BNK5⁺ show both fluorescence and phosphorescence, intensities are quite weak. Therefore, our attention turned to the preparation of solid-phase complexes that luminesce more than 2 orders of magnitude more strongly. Most of the experiments were performed with 5-(4-bromo-1-naphthoyl)pentyltrimethylammonium bromide (BNK5⁺), which is similar spectrally to BNK10⁺. Figure 7 shows the luminescence emission spectrum for solid-phase BNK5⁺ on γ -CD modified cellulose resin. Solid-phase luminescence spectra for BNK5⁺ are similar to solution spectra for BNK10⁺ (including the difference in excitation spectra for phosphorescence and fluorescence) except that the phosphorescence splits into two partially resolved bands. Although the maxima in the excitation spectra are in the ultraviolet region, observable fluorescence and phosphorescence can be excited from solid-phase BNK reagents at 370 nm, making this system compatible with glass fibers and an incandescent source.

Solid-phase BNK5⁺ on γ -CD modified cellulose phosphoresces in the presence of oxygen. As shown in Figure 5, the Stern-Volmer plot for oxygen quenching of solid-phase BNK5⁺ phosphorescence is nonlinear, suggesting that BNK5⁺ exists in at least two states which differ in their susceptibility to oxygen quenching. This agrees with literature data showing that the phosphorescence includes a short lifetime component susceptible to oxygen quenching and a longer lived component unaffected by oxygen (12).

Of the systems studied, solid-phase BNK5⁺ on β -cyclodextrin cellulose is best suited for determining oxygen levels up to about 20% where the slope of the Stern-Volmer plot decreases. It allows a luminescence ratio measurement with excitation close to the edge of the visible spectral region. Furthermore, fluorescence and phosphorescence intensities are quite stable to continuous photoexcitation, decreasing only 4% after 3 h of continuous illumination.

Unfortunately, however, this reagent is not suitable for analysis of aqueous samples. Although reversible response is observed for gas-phase samples bubbled through water, wetting of the reagent causes a drastic decrease in phosphorescence intensity. This is observed not just for direct wetting of the sample but upon exposure of the reagent to an

atmosphere with 100% relative humidity for 2 days. Absorption spectra of water that had wetted γ -CD/BNK5⁺ showed that at least part of the problem is extraction of the lumophore from the CD cavity. Although the reagent in the oxygen sensor is separated from the sample by a hydrophobic membrane, it is necessarily exposed to 100% relative humidity since membranes used in oxygen sensors all have some degree of permeability to water vapor.

Since the problems with aqueous samples was thought to be due to extraction of the chromophore from the CD cavity, efforts were made to modify the reagent to reduce its extractability. One approach was to exchange the bromide of BNK5⁺ to a more hydrophobic counterion in the hopes of forming an ion pair that would not be readily extracted. However, the four anions tried, 2-bromoethanesulfonate, 12-bromododecanoate, poly(styrene)sulfonate, and stearate, all failed to modify the effect of wetting.

A second approach was to use nonionic bromonaphthoyl derivatives which would have lower water solubilities and thus would be expected to have stronger tendencies to bind within the hydrophobic CD cavity. An inclusion complex of 5-(4-bromo-1-naphthoyl)-1-pentanol (BNK5OH) in γ -cyclodextrin cellulose photodecomposed rapidly, while an inclusion complex of 1-(4-bromo-1-naphthoyl)heptadecane (BNK17) in γ -CD showed oxygen-insensitive fluorescence and oxygen-sensitive phosphorescence. As shown in Figure 5, the Stern-Volmer plot for the latter compound is almost linear with reasonable sensitivity to variations in oxygen partial pressure. However, the phosphorescence from this compound was reduced in the presence of aqueous samples. Since BNK17 is far too hydrophobic to have any appreciable water solubility, it indicates that the phosphorescence quenching effect of water must go beyond simple extraction of the lumophore from the CD cavity. Apparently, hydration of the CD is sufficient to render included lumophores susceptible to efficient quenching. It should also be kept in mind that in the case of BNK17, the CD cavity may contain the hydrocarbon tail rather than the bromonaphthoyl group.

CONCLUSIONS

We have succeeded in demonstrating the principle that oxygen can be measured by using an indicator that is based on a ratio of luminescence intensities. Furthermore, we have developed an indicator, BNK5⁺ on γ -CD cellulose, that has sufficient sensitivity and stability to be useful for measuring gas-phase oxygen concentrations in atmospheres with low relative humidity. However, we have yet to come up with a practical ratio-based indicator system to measure oxygen in aqueous samples.

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Rapid Rate Measurements by the Pulsed-Accelerated-Flow Method

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First-order rate constants as large as $124\,000\text{ s}^{-1}$ ($t_{1/2} = 5.6\text{ }\mu\text{s}$) and second-order rate constants as large as $2 \times 10^9\text{ M}^{-1}\text{ s}^{-1}$ are measured with a pulsed-accelerated-flow (PAF) spectrometer. The method uses a variation of flow velocity during data collection to resolve reaction rate constants from mixing rate constants. The validity of the method is demonstrated by calibration reactions under pseudo-first-order and second-order (equal and unequal concentration) conditions. The performance and limitations of a twin-path mixing/observation cell and the PAF method are reported.

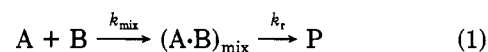
Many irreversible reactions are too fast to study by stopped-flow methods (1-3) and cannot be studied by relaxation methods, which require reversibility. A recent paper (4) described a pulsed-accelerated-flow (PAF) spectrophotometer that measured pseudo-first-order rate constants in the range of $300\text{--}12\,000\text{ s}^{-1}$ and consumed only 6 mL of each reagent per push. In the present work we extend the range of pseudo-first-order rate constants that can be measured by the PAF method to include $200\text{--}124\,000\text{ s}^{-1}$. We also show that second-order rate constants as large as $2 \times 10^9\text{ M}^{-1}\text{ s}^{-1}$ (values that approach the diffusion controlled limit) can be determined.

The PAF method is based on the continuous flow method with integrating observation (CFMIO), in which the reaction is observed along the direction of flow from the point of mixing to the exit of the solution from the observation tube (2, 3, 5). The observed absorbance is a function of the rate of mixing, the rate of chemical reaction, the length of the observation tube, and the flow velocity, as well as the molar absorptivities and concentrations of the reactants and products. Gerischer and Heim (5, 6) derived equations that describe the absorbance as a function of velocity for a CFMIO experiment when mixing is ideal. These equations are given in Table I for first-order and second-order (equal and unequal concentration) reactions.

In the PAF method solutions experience a constant acceleration during each push and, therefore, have a range of flow velocities. Figure 1 illustrates the linear velocity ramp that permits 250 instantaneous velocities between 3 and 11 m s^{-1} to be sampled for solutions in the observation tube. All velocities in the range are large enough to give turbulent flow in the observation tube (2, 3). We have shown for first-order conditions that the absorbance measured in a constant velocity

experiment is equal to the absorbance at the same instantaneous velocity in a PAF experiment (4). For the time interval of each measurement, the solution in the observation tube experiences a very small range of velocities, and therefore the reaction profile is similar to that developed during a constant velocity experiment.

The equations in Table I apply to systems where the mixing process can be neglected. This requires complete mixing in a segment of the observation tube that is negligible compared to the length of the tube required for the reaction. Faster chemical reactions are complete within shorter segments of the observation tube so that this assumption is not valid. Hence, for fast reactions the mixing process results in a bias in the measured absorbance values. An apparent rate constant (k_{app}), which is less than the actual rate constant, can be calculated by using the ideal equation and the measured absorbance values. We have demonstrated (4) that both a mixing rate constant ($k_{\text{mix}},\text{ s}^{-1}$) and a first-order reaction rate constant ($k_r,\text{ s}^{-1}$) can be resolved from k_{app} based on successive first-order processes (eq 1). The first step is the physical mixing



of the reactants A and B. The mixing rate constant depends on the flow velocity, $v\text{ (m s}^{-1}\text{)}$, in the mixing/observation tube where k_m is a proportionality constant (m^{-1}) (eq 2). The

$$k_{\text{mix}} = k_m v \quad (2)$$

second step is the chemical reaction rate process of the mixed reactants, $(A \cdot B)_{\text{mix}}$, to give products (P). Previously, pseudo-first-order conditions were used with one of the reactants in large excess. Equation 3 gives the double-reciprocal de-

$$\frac{1}{k_{\text{app}}} = \frac{1}{k_m v} + \frac{1}{k_r} \quad (3)$$

pendence that is found experimentally. Plots of $1/k_{\text{app}}$ vs. $1/v$ are linear with the intercepts equal to $1/k_r$ and the slopes equal to $1/k_m$. In the present work we demonstrate that eq 3 also can be applied to reactions studied under second-order equal and unequal concentration conditions.

In this work a new twin-path mixer with 10 radial input jets is developed. The point of mixing is at the center of the cell, and the reactants flow in opposite directions away from this point. Consequently, light passes through twin segments of identical mixing and reaction profiles. This mixing cell is used to study reactions under pseudo-first-order conditions