Fluorescence Quenching Method for Determining Equilibrium Constants for Polycyclic Aromatic Hydrocarbons Binding to Dissolved Humic Materials

Thomas D. Gauthier,1 Edward C. Shane,1,4 William F. Guerin,1 W. Rudolf Selz,1,4 and Clarence L. Grant1,4

Department of Chemistry and Department of Microbiology, University of New Hampshire, Durham, New Hampshire 03824

A fluorescence quenching method has been developed for determining equilibrium constants for the association of pyrene, phenanthrene, and anthracene with dissolved humic and fulvic acids. The technique is based upon the observation that polycyclic aromatic hydrocarbon (PAH) fluorescence in aqueous solution is quenched upon association with humic material. Association constants are derived from the fractional decrease in fluorescence intensity as a function of added humic material with Stern-Volmer plots. This allows association constants to be measured without a separation. In addition, this technique permits a ratio measurement so the exact concentration of pollutant need not be determined. Stern-Volmer plots were linear for the three PAHs studied. Anthracene–humic association constants determined by the fluorescence quenching technique correlated reasonably well with values determined by a reverse-phase method. However, unlike the reverse-phase method the measured association constants do not depend on the concentration of dissolved organic matter.

Introduction

The importance of dissolved organic matter (DOM) in determining the fate of hydrophobic organic pollutants in aqueous environments has already been shown (1–5). Several researchers have reported the affinity of various pollutants for DOM, and they suggest that upon association rates of chemical and biological degradation, photolysis, and volatilization may be altered. In the past, DOM has usually been neglected as an environmental compartment in the development of predictive transport and fate models. However, Caron et al. (4) have shown that for DDT the fraction of pollutant associated with DOM is greater than the fraction associated with suspended sediments or biota. Although its relative concentration in aquatic systems may be low, DOM can represent a large fraction of the total sorptive capacity of the system due to the large volume of solution.

Recently, DOM has also been implicated in the apparent anomalous effect of solids concentration on the sorptive partitioning of hydrophobic pollutants to suspended sediments and soils (6–8). One possible explanation for the decrease in observed partition coefficients as the solids-to-solution ratio increases can be based on changing concentrations of DOM.

Described here is a new method to measure polycyclic aromatic hydrocarbon (PAH) binding to DOM. The method is based upon the observation that PAHs fluoresce in aqueous solution but not when associated with DOM as represented by dissolved humic and fulvic acids. As a consequence, the fraction of PAH associated with DOM may be determined directly from the fractional decrease in fluorescence upon addition of humic substance.

For compounds that fluoresce, this method offers advantages over previous methods that depend on the separation of free from bound pollutant. Separation has been achieved via dialysis (9), adsorption on XAD-2 resin (10), ultrafiltration (11–13), gel permeation chromatography (14, 15), and reversed-phase chromatography using C-18 Sep-Pak cartridges (16). Often the fraction of bound pollutant is estimated indirectly from the difference between the initial and final concentrations of free pollutant. In view of the difficulties in separating complex natural organic substances, it seems inevitable that such difference measurements have large uncertainties. There is a concern that the act of separating free from bound pollutants may disrupt equilibria and lead to variable estimates of binding constants. In the fluorescence quenching experiment, there is no separation step involved, and the initial concentration of pollutant need not be determined. Consequently, this technique offers potential improvements in reproducibility and convenience for fluorescent compounds. Fluorescence quenching has been previously used to follow the rate of association between PAHs and dissolved humic matter (5).

Fluorescence polarization has been used to study the association of perylene with fulvic acid in glycerol/water mixtures (17), but this technique is only applicable in systems where the fluorescence is not quenched by the presence of humic material.

Experimental Section

Apparatus. Fluorescence measurements were made on a Perkin-Elmer MPP-44E spectrofluorometer with slit widths set for band widths of 2 nm on both excitation and emission monochromators. Fluorescence was measured as a function of added humic material at a fixed wavelength. The excitation/emission wavelengths used for the three PAHs studied were 272/371 nm for pyrene, 250/380 nm for anthracene, and 288/364 nm for phenanthrene.

Absorbance measurements were made on a Bausch and Lomb Spectronic 200 recording spectrophotometer. A Beckman LS-7000 liquid scintillation counter equipped with automatic quench compensation was used to measure radioactivity. The carbon, hydrogen, and nitrogen contents of humic materials were determined on a Perkin-Elmer 240B elemental analyzer.

Materials. The method was tested for three different PAHs with three fulvic acids and three humic acids, each of which was fully characterized. One fulvic acid, designated SW, was isolated from a podzolic soil originating in North Conway, NH. Its characteristics have been published (18, 19). A second fulvic acid, designated AB, was isolated from a podzolic soil originating in Lee, NH, by acid extraction and purified by repeated passage over Amberlite IR 120 cation exchange resin (20). The third fulvic acid, designated SR, was extracted from the Suwannee River in southeastern Georgia; it has been comprehensively characterized (21). One humic acid, designated AB, was isolated from a podzolic soil in Lee, NH, by treatment with dilute base. Following acidification to pH 1 with HCl, it was purified by five successive extractions with 2% HF/0.5% HCl. A second humic acid, designated TG, was

1 Contribution No. UNH-MP-JR-SG-86-1.
2 Department of Chemistry.
3 Permanent address: Department of Chemistry, Morningdale College, Sioux City, IA 51106.
4 Department of Microbiology.
isolated from a dark lignite soil in the same manner. The third humic acid was obtained from Aldrich and used without further purification. Elemental and functional group analyses are summarized in Table I. Concentrated fulvic acid solutions were prepared by dissolving weighed amounts in water with the aid of ultrasonic agitation. Dissolution of humic acid required temporary elevation of pH. A working pH was then obtained by acidification with HCl when necessary.

The PAHs used were pyrene (Aldrich, red label, 99+% pure), phenanthrene (Aldrich, red label, 98+% pure), and anthracene (Aldrich, gold label, 99.9+% pure). All were used without further purification. PAH solutions below the solubility limit were prepared by dissolving weighed amounts in water with the help of ultrasonic agitation (usually 24–28 h). The solutions were stored in glass volumetric flasks in the dark. Adsorption of PAH to the walls of containers made it impractical to know the exact concentration of material in solution; however, it should be noted that an accurate PAH concentration is not required by the fluorescence quenching method.

Stock 0.10 M buffer solutions were prepared with sodium acetate and acetic acid in the proportions necessary to achieve the desired pH. These stock solutions were diluted by a factor 10 when used to buffer PAH solutions. A phosphate buffer with the same ionic strength as the acetate buffer was used to obtain a pH of 6.0. Distilled deionized water was used for all solutions.

Radiolabeled [9(10)-14C]anthracene (15.1 mCi/mmmole) was obtained from Amersham and purified by preparative thin-layer chromatography before use. Column separations required to determine partition coefficients by use of the radiolabeled anthracene were made with C-18 Sep-Pak cartridges (Waters Associates).

**Procedures.** A typical experiment involved preequilibrating 2.25 mL of aqueous PAH solution, 0.250 mL of the appropriate aqueous buffer solution, and a micro stir bar in a 1 × 1 × 4 cm quartz fluorescence cuvette for a period of 30 min. By monitoring the initial decrease in fluorescence intensity as a function of time for a period of 1 h, it was determined that 30 min was an adequate equilibration period so that further adsorption to the walls of the cuvette was negligible. An initial fluorescence intensity value was then recorded. Following this, a 10-μL aliquot of a 500 ppm stock humic acid solution was added to the cuvette. Equilibration of the PAH was rapid, requiring less than 1 min. However, the solution was stirred for 3–4 min and then allowed to stand quiescent for a minute before a second fluorescence intensity value was recorded. This step was repeated until a total of five to six aliquots of humic material had been added to the cuvette, bringing the total concentration of humic material to 12–16 ppm. At this concentration, the fluorescent intensity of the initial PAH solution had decreased by approximately 50%, implying that approximately 50% of the PAH was associated with humic material. The fulvic acids were found to have weaker association constants and thus required larger aliquots and a final concentration of 28–30 ppm before an equivalent decrease in fluorescent intensity was observed.

While humic material was being added to solutions of PAH and during equilibration, the shutter of the spectrofluorometer was closed to protect the PAH from photodegradation. The shutter was only opened for the actual intensity measurements. Since each intensity measurement required 1 min, the total time of exposure to UV radiation was 5–6 min. In experiments in which PAH in the presence of humic material was continuously irradiated with UV radiation, no decrease in intensity was observed, indicating that significant photodegradation does not occur during a binding constant measurement sequence.

An alternate procedure was also developed in which 200–400-μL aliquots of humic or fulvic acid were added to 50 mL of buffered PAH solution in a Teflon bottle. After equilibration, some of the solution was transferred to a quartz cuvette for the fluorescence measurement and then returned to the Teflon bottle. This procedure gave similar results, but it required an added transfer step, which was cumbersome to perform. It was, therefore, abandoned in favor of the previously described method.

Absorbance measurements required to correct for inner filter effects were taken after each aliquot of humic material had been added during an actual experiment or at a later date on a solution of identical composition. In some samples, the measured fluorescence included a background component from the humic material. To correct for this, the fluorescence of a solution containing humic substance only was measured with both the same concentrations and the same instrumental conditions as for the measurements with humic substance and PAH together. The fluorescence intensity measured for humic substance only was subtracted from the total fluorescence intensity measured for PAH in the presence of humic substance before proceeding with data analysis. In the experiments reported here, the maximum background was only 3% of the total. However, to maximize PAH fluorescence relative to background, it is recommended that PAH concentrations at or near the solubility limit be used when this method is applied. The background may be more significant when the fluorescence quenching method is applied with PAHs that have lower water solubilities than anthracene, pyrene, and phenanthrene. (Note, however, that PAHs with lower solubilities tend to bind more strongly to humic substances so that the humic concentrations required for the binding constant measurement are lower.)

The comparison method for obtaining partition coefficients using C-18 Sep-Pak cartridges has already been described (16). The method was carried out as reported in the literature, including correction for background when samples were counted by liquid scintillation spectrometry.

**Data Treatment.** The fluorescence quenching technique is based upon the observation that the intensity of fluorescence is proportionately decreased upon the addition of humic or fulvic acids. The association of PAH with humic or fulvic acids may be represented by the following equations:

\[
\text{PAH} + \text{Hu} \leftrightarrow \text{PAH-Hu}
\]

\[
K_D = \frac{[\text{PAH-Hu}]}{([\text{PAH}][\text{Hu}])}
\]
where PAH = polycyclic aromatic hydrocarbon, Hu = humic substance, PAH–Hu = humic-associated PAH, and $K_0$ is the association constant. The mass balance on the PAH is described in eq 3, where $C_{PAH}$ is the formal or total concentration of PAH. Combining eq 2 and 3 and rearranging yields eq 4. If we assume that the fluorescence

$$C_{PAH}/[PAH] = 1 + K_0[Hu]$$

(4)

intensity is proportional to the concentration of free PAH in solution ([PAH]), then

$$F_0/F = 1 + K_0[Hu]$$

(5)

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of humic material, respectively. Since, at the concentrations used, a significant excess of humic acid was present, [Hu] was taken as the amount of added humic without correction for the fraction of humic that was associated with PAH. Equation 5 is in the form of the Stern–Volmer equation.

In general, there are three different types of quenching processes that can occur: static, dynamic, and apparent. In static quenching, a nonfluorescent complex is formed between the fluorophore and quencher. Equations 1–5 represent a simple derivation of the Stern–Volmer equation resulting from static quenching. An alternative quenching process, also adequately described by the Stern–Volmer equation, is dynamic or collisional quenching. In dynamic quenching, the quencher must diffuse to the fluorophore during the lifetime of its excited state and nonradiatively deactivate that state. For dynamic quenching, the Stern–Volmer quenching constant is equal to the product of the bimolecular quenching rate constant and the fluorescent lifetime of the fluorophore in the absence of quencher. This imposes constraints on the size of the Stern–Volmer constant as the bimolecular quenching rate constant has a diffusion-controlled limit. The reader is referred elsewhere for a complete derivation of the Stern–Volmer equation for dynamic quenching (22, 23).

Apparent quenching is not a quenching process at all but is rather due to an attenuation of the excitation beam and/or absorption of emitted radiation by an excess concentration of fluorophore or by the presence of an additional absorbing species in solution. This phenomenon is more commonly known as the "inner filter effect". Corrections can be made by taking into consideration the cell geometry and absorption characteristics of the solution. The higher concentrations of humic and fulvic acids used in the fluorescence quenching titrations absorbed light to a significant extent at both excitation and emission wavelengths. It was therefore necessary to correct for this effect on the basis of the cell geometry shown in Figure 1 and the absorption characteristics of the solution as described by Parker in the following equation (22, 24):

$$\frac{F_{cor}}{F_{obsd}} = \frac{2.3dA_{ex}}{1 - 10^{-dA_{em}}}$$

(6)

where $F_{obsd}$ is the observed intensity, $F_{cor}$ is the corrected intensity, and $A_{ex}$ and $A_{em}$ are the absorbances per centimeter at the excitation and emission wavelengths, respectively. The remaining terms, $d$, $s$, and $g$, depend upon the geometry of the measurement and are defined in Figure 1. The maximum value of the correction factor did not exceed 1.8, which is well within the recommended acceptable range.

Results and Discussion

Table II gives the concentrations of fulvic acid, absorbance readings, and fluorescent intensities after each addition of titrant for the interaction of phenanthrene with 4AB fulvic acid. The inner filter effect correction factor at each fulvic acid concentration is also calculated. Fluorescent intensities corrected for dilution effects and the inner filter effect are listed under the heading $F_{cor}$. The resulting Stern–Volmer plot of $F_0/F$ values vs. [FA] is found in Figure 2 along with the calculated slope.

Table III illustrates the variation of slopes for Stern–Volmer plots for the interaction of three different PAHs with the 1AB humic acid. Table IV shows the effect of three different fulvic acids and one humic acid on the fluorescence of pyrene. In all cases, after correction for inner filter effects and background fluorescence, the interaction of humic or fulvic acid with the three PAHs studied resulted in linear Stern–Volmer plots.

Since both static and dynamic quenching can be described by linear Stern–Volmer plots, the data were ana-
lyzed to determine which type of quenching was being observed. The measurement of fluorescent lifetimes in the presence and absence of quencher is the most definitive method to distinguish static and dynamic quenching. However, as we were unable to obtain lifetime measurements, we chose to evaluate the data under both conditions. Analysis of the data in Table II for the interaction of phenanthrene with 4AB fulvic acid resulted in the Stern–Volmer quenching constant ($K_s$) of $3.2 \times 10^4$ mL/g. Assuming a molecular mass of 500 g/mol for the fulvic acid, $K_s$ becomes $1.6 \times 10^4$ M⁻¹. The fluorescence lifetime for phenanthrene in the absence of quencher is $5.0 \times 10^{-8}$ s (25). Since, for a dynamic quenching process, $K_s$ is equal to the bimolecular quenching rate constant, $K_q$, times the fluorescence lifetime in the absence of quencher, the bimolecular quenching rate constant can be calculated. In this case, $K_q = 3.2 \times 10^{-11}$ M⁻¹ s⁻¹, which is unreasonably high for a diffusion-controlled process in aqueous solution. A bimolecular quenching constant around $1 \times 10^{-9}$ M⁻¹ s⁻¹ is the largest possible value in aqueous solution (23). Analysis of other data sets leads to similar conclusions indicating that at most only a tiny fraction of the observed quenching can be dynamic.

If the Stern–Volmer quenching constants are interpreted as ground-state association constants for the PAH binding to humic material, then $K_s = K_a$. For the above system, $K_a = 3.2 \times 10^4$ mL/g. Dividing $K_a$ by the fraction of organic carbon in the sorbing material yields a $K_{so}$ value of $7.7 \times 10^4$ mL/g, where $K_{so}$ is the association constant normalized to organic carbon content. This value is well within the range of values reported by other researchers for the association of PAH to DOM. With the fluorescence quenching technique to follow anthracene binding to a variety of humic materials, $K_{so}$ values varied from $1 \times 10^4$ to $6 \times 10^4$ mL/g. Landrum reported values of $(1-8) \times 10^4$ mL/g for anthracene binding to humic material in aqueous solution using a reverse-phase method and values of $(3-50) \times 10^4$ mL/g using a dialysis technique (16). Means and Wijayaratne reported a value of $51 \times 10^4$ mL/g for anthracene binding to estuarine colloids (11). In view of this agreement with other methods, we believe that the Stern–Volmer quenching constants are best interpreted as ground-state association constants and not as bimolecular quenching rate constants. The same conclusion was drawn by Patonay et al. (26), who used fluorescence quenching to follow the interaction between pyrene and cyclodextrin.

The fact that Stern–Volmer plots are linear allowed us to rule out other possible mechanisms. If two fluorophores are present and one is not accessible to quencher, then the plot will deviate from linearity toward the $x$ axis. If the quenching process is actually a combination of both static and dynamic quenching, then the plot deviates from linearity toward the $y$ axis (23). The possibility that PAHs bound to humics also fluoresce but with a lower efficiency was also considered. However, this too would produce a nonlinear Stern–Volmer plot.

With use of the fluorescence quenching technique, under constant pH and ionic strength conditions, $K_{so}$ values can be determined with a reproducibility of 3.3% RSD (27). This value was calculated from three fractional experiments performed in duplicate and in random order over a period of 6 weeks. However, under variable pH and ionic strength conditions, $K_{so}$ values varied by as much as a factor of 2.5, depending upon the humic or fulvic acid used and the ionic species in solution. For the interaction of pyrene with 1AB humic acid, the calculated $K_{so}$ value was found to vary by a factor of 2 when the ionic strength was varied from 10⁻⁴ to 0.5 M. If sodium chloride was used as the ionic strength determining species, then $K_{so}$ increased with increasing ionic strength. If an acetate buffer was used to control the ionic strength, then the effect was in the opposite direction. The effect of pH was not as dramatic. The $K_{so}$ values decreased only slightly when the pH was increased from 5 to 8.

### Comparison with Reverse-Phase Method

In an attempt to further confirm the validity of the fluorescence quenching technique, $K_{so}$ values for the interaction of anthracene with five different humic and fulvic acids were determined under the same pH and ionic strength conditions by using both fluorescence quenching and the reverse-phase separation technique described by Landrum et al. (16). Table V shows the results.

It is difficult to directly compare the two methods since the values measured by the reverse-phase method vary in a systematic way with DOM concentration. Landrum et al. reported $K_{so}$ values to decrease by a factor of 3.1 as the concentration of DOM increased over a similar range (16). If $K_{so}$ values really were changing, this would be reflected in nonlinear Stern–Volmer plots, which are not observed. Landrum et al. suggested that the variation in $K_{so}$ with DOM that they observed could be due to humic acid interactions. However, in view of the fact that the simpler fluorescence quenching technique does not show the same effect plus the fact that humic and fulvic acids do not aggregate at the concentration levels and pHs used in this study, we believe the variation in $K_{so}$ with DOM reported by Landrum et al. to be an artifact of this method. (It is also worth noting that values measured by the reverse-phase separation technique vary with flow rate, an observation that is highly suggestive of artifacts.)

<table>
<thead>
<tr>
<th>Table V.</th>
<th>$K_{so}$ Values for the Interaction of Anthracene with DOM*</th>
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<tbody>
<tr>
<td>DOM</td>
<td>Fl. quenching</td>
</tr>
<tr>
<td>SW FA</td>
<td>1.6</td>
</tr>
<tr>
<td>AB FA</td>
<td>3.2</td>
</tr>
<tr>
<td>Aldrich</td>
<td>5.2</td>
</tr>
<tr>
<td>AB HA</td>
<td>5.7</td>
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<tr>
<td>TG HA</td>
<td>8.4</td>
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</table>

* $K_{so}$ values are expressed $\times 10^4$ mL/g. The range of humic concentrations used in determining the $K_{so}$ by fluorescence quenching runs from 0 to 9 ppm except for SW FA, where it is from 0 to 17 ppm. The four values listed under reverse phase were measured at 4.8, 9.6, 14.4, and 19.2 ppm humic material, respectively, except for AB FA where the humic concentrations were 6.0, 11.9, 17.3, and 22.8 ppm.
In general, the two methods show reasonable agreements. At a DOM concentration of 4.8 ppm, $K_{eq}$ values by the reverse-phase separation procedure for the three humic materials are in excellent agreement with the fluorescence quenching values, while estimates for the two fulvic acids differ by a factor of 2. At this concentration, the low correction coefficient ($r = 0.57$) is primarily due to the poor agreement for the fulvic acids. At a DOM concentration of 19.2 ppm, the fluorescence quenching values exceed those of the reverse-phase method by a factor of 2 for the fulvic acids and a factor of 3 for the humic acids. The correlation coefficient, however, increases to a value of 0.87.

The fluorescence quenching technique can only be applied to measure $K_{eq}$ values for compounds with high fluorescence efficiencies, notably PAHs. However, where applicable, it offers significant advantages. The major advantage is that no separation step is required, thus eliminating possible errors due to incomplete separation of free from bound pollutant. The technique of fluorescence is inherently sensitive, enabling measurements of very low pollutant concentrations. Furthermore, the technique involves a ratio measurement so that it is not necessary to know the exact pollutant concentration. This is especially useful since most hydrophobic organic pollutants are only very slightly soluble in aqueous solution, making it difficult to prepare solutions of accurately known concentrations. The technique is relatively rapid (complete analysis in less than 1 h) and offers very good precision.

In principle, the fluorescence quenching approach can be applied with dissolved organic matter directly in natural samples. However, if this is attempted, it should be confirmed that there is no quenching due to components of the natural sample other than the dissolved organic matter. We feel that the fluorescence quenching method will have its greatest value in determining how factors such as humic matter structure, ionic strength, pH, etc. affect $K_{eq}$ values.

Registry No. Pyrene, 129-90-0; phenanthrene, 85-01-8; anthracene, 120-12-7.

Literature Cited


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