

RADIATION BOUNDARY CONDITIONS IN COLLISIONAL QUENCHING OF FLUORESCENCE; DETERMINATION BY FREQUENCY-DOMAIN FLUOROMETRY

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Quenching of 1,2-benzanthracene (BA) by CBr₄ was studied by frequency-domain fluorometry. The frequency response of the BA emission could not be fit using the Smoluchowski decay law for transient effects in quenching, $\exp(-t/\tau - 2bt^{1/2})$. However, the data were consistent with the expression for the radiation boundary conditions. We believe these are the first reported data which support the "radiation boundary condition" model for collisional quenching. Our ability to detect the inadequacy of the $t^{1/2}$ model indicates that the frequency-domain method can yield considerable detail about diffusive processes in solution.

1. Introduction

Collisional quenching of fluorescence requires contact between the fluorophore and quencher during the lifetime of the excited state [1]. In the presence of quenching the decays of fluorescence intensity are expected to become more complex than a single exponential [2] due to transient effects which occur immediately following excitation of the fluorophore. The detailed form of the decay law is expected to depend upon the interaction radius (R), the sum of the donor and acceptor diffusion coefficients (D) and the specific rate constant (κ) for quenching [3-7]. Consequently, the time-resolved decays can potentially reveal considerable detail of diffusive processes in solution, which in turn can be compared with various theories [8-11].

To date there have been relatively few studies of transient effects in quenching. Most of these studies have been performed in the time domain [5-7,12]. The data were adequate to detect the quenching-induced complexity in the decay laws. The time-resolved data were fit to an approximate form of the expected decay, $\exp(-t/\tau - 2bt^{1/2})$. For the present studies we used the alternative method of frequency-domain fluorometry [13-15]. In this method time-

resolved parameters are recovered from the frequency response of the emission to intensity-modulated light. The frequency-domain method is now known to provide high resolution of complex emission processes. In preliminary experiments on quenching we found that decay laws of the form $\exp(-t/\tau - 2bt^{1/2})$ were easily detected in the presence of quenching [16], even in solutions with low viscosities and displaying short decay times near 4 ns. We also noticed that this decay law could not account for the data at higher quencher concentrations.

In the present report we describe our examination of the quenching of 1,2-benzanthracene (BA) by carbon tetrabromide (CBr₄). This system was described previously in the pioneering reports by Ware and co-workers [6,7]. With the resolution provided by the frequency-domain data we found that the approximate decay law of the form $\exp(-t/\tau - 2bt^{1/2})$ was completely inadequate to account for the complexity in the BA decay which was induced by quenching. However, the frequency-domain data were consistent with the more complete expression based on the "radiation" boundary conditions [3,4,7]. To the best of our knowledge this is

the first report in which this more complex decay law has been detected.

2. Theory

Consider a fluorophore which displays a single decay time (τ_0) in the absence of quenching. Then, in the presence of quenching the population of excited fluorophores is given by the solution of

$$dI(t)/dt = -\{t/\tau_0 + k(t)[Q]\}I(t), \quad (1)$$

where $[Q]$ is the concentration of quencher and $k(t)$ is the time-dependent quenching constant. The simplest model is to assume $k(t)$ is not time dependent, in which case the quenching constant is given by

$$k = 4\pi RN'D. \quad (2)$$

In this equation the efficiency of quenching is assumed to be unity, R is the interaction radius or the sum of the radii of the fluorophore (F) and the quencher (Q), D is the sum of their diffusion coefficients and $N' = 6.02 \times 10^{20}$. Since the decays of BA in the presence of quencher were found to deviate from a single exponential this model can be rejected.

The next case is to assume that the fluorophore is immediately quenched when a quencher reaches the distance R . This results in a step function increase in quencher concentration from 0 to $[Q]$ around the excited fluorophores. Then, the time-dependent rate constant is given by

$$k(t) = 4\pi DN'R[1 + R(\pi Dt)^{-1/2}] \quad (3)$$

as described originally by Smoluchowski [2]. Solving eq. (1) for the intensity decay yields

$$I(t) = I_0 \exp(-t/\tau - 2bt^{1/2}), \quad (4)$$

where

$$1/\tau = 1/\tau_0 + 4\pi RDN'[Q], \quad (5)$$

$$b = 4R^2N'(\pi D)^{1/2}[Q]. \quad (6)$$

In an elegant paper Collins and Kimball [3] (see also ref. [4]) pointed out that eq. (3) cannot be completely accurate since $k(t)$ becomes infinite at $t=0$. To avoid this difficulty they softened the concentration gradient by assuming the rate of quenching is dependent upon the F-Q distance. This is called

the "radiation" boundary condition, and results in more complex form for the quenching constant,

$$k(t) = \frac{4\pi RDN'}{1 + D/\kappa R} \times [1 + (\kappa R/D) \exp(x^2) \operatorname{erfc}(x)], \quad (7)$$

$$x = [(Dt)^{1/2}/R](1 + \kappa R/D). \quad (8)$$

This expression introduces a new parameter κ , which is a specific rate constant for quenching and has units of cm/s. The specific rate constant can be converted to the more familiar units ($M^{-1} s^{-1}$) by forming product $4\pi R^2N'\kappa$. The intensity decay is given by

$$I(t) = I_0 \exp(-t/\tau_0) \times \exp\left(-[Q] \int_{t=0}^t k(t) dt\right). \quad (9)$$

The important point is that eq. (4) is thought to be only approximately correct, whereas eqs. (8) and (9) should provide a more accurate description of the intensity decays.

3. Frequency-domain theory and analysis

We obtained the time-resolved information from the frequency response of the emission to amplitude modulated light, which is characterized by the frequency (ω) dependent values of the phase shift ($\phi_{c\omega}$) and the extent of demodulation ($m_{c\omega}$). The parameters describing the decay law are compared with the calculated (c) values ($\phi_{c\omega}$ and $m_{c\omega}$). For any decay law these values are given by

$$\phi_{c\omega} = \arctan(N_{c\omega}/D_{c\omega}), \quad (10)$$

$$m_{c\omega} = (N_{c\omega}^2 + D_{c\omega}^2)^{1/2}, \quad (11)$$

where

$$N_{c\omega} = \int_0^\infty I(t) \sin(\omega t) dt \Big/ \int_0^\infty I(t) dt, \quad (12)$$

$$D_{c\omega} = \int_0^\infty I(t) \cos(\omega t) dt \Big/ \int_0^\infty I(t) dt \quad (13)$$

and ω is the modulation frequency in radian/s. The goodness-of-fit is characterized by

$$\chi_R^2 = \frac{1}{\nu} \sum_{\omega} \left(\frac{\phi_{\omega} - \phi_{c\omega}}{\delta\phi} \right)^2 + \frac{1}{\nu} \sum_{\omega} \left(\frac{m_{\omega} - m_{c\omega}}{\delta m} \right)^2, \quad (14)$$

where ν is the number of degrees of freedom, and $\delta\phi$ and δm are the experimental uncertainties in ϕ_{ω} and m_{ω} . We used values of 0.2° and 0.005 , respectively, which were found to be appropriate for our instrument and measurement techniques. The parameters were determined by the method of non-linear least squares [17,18].

For the $t^{1/2}$ -dependent decays (eq. (4)) we used numerical integration to evaluate the sine and cosine transforms (eqs. (12) and (13)). In particular, we used an adaptive Newton-Cotes 9-point integration [19], with care to match the time range of integral to that of the intensity decay. It is important to use small time increments in the early portion of the decay because of the initial rapid decay due to the transient terms. For the radiation boundary conditions (eqs. (7)–(9)) we used a similar routine, except that the term

$$f(t) = \int_0^{t=0} \exp(x^2) \operatorname{erfc}(x) dt$$

was obtained during the iterations from a look-up table.

4. Materials and methods

The frequency-domain data were obtained using the instrument described previously [13]. The excitation source was the 325 nm output from HeCd laser, modulated with an electro-optic modulator (Lasermetrics 1042). The polarizers were oriented to yield rotation-free data. The emission was observed through a Corning 0-52 filter.

1,2-benzanthracene was obtained from Starks Associates, Inc., Buffalo, NY, Lot ET10-85-1, CBr₄ from Eastman Organic Chemicals, reagent grade propylene glycol from Sigma, and were used without further purification. Samples were purged with

nitrogen to remove dissolved oxygen. Examination of blank samples revealed no detectable impurities. The purity of the BA and other reagents is supported by the single exponential decays found in the absence of quenching and the agreement of the decay times with those reported previously [7]. The extent of quenching with 340 nm excitation were in agreement with those reported by Nemzek and Ware [7], following correction for the optical density of CBr₄ at 340 nm.

5. Results

The frequency-response of the BA emission was examined in the absence and presence of CBr₄. In the absence of quenching by CBr₄ the decays were found to be single exponentials. This is seen from the low value of χ_R^2 for the single exponential fits (table 1) and by the agreement between the data (●) and the single exponential model (—, fig. 1). Also, the values of these decay times are in precise agreement with those obtained previously using time-correlated

Table 1
Multi-exponential decay analysis of 1,2-benzanthracene in propylene glycol quenched by CBr₄

[CBr ₄] (M)	T (°C)	τ_i (ns)	α_i	f_i	χ_R^2
0	15	39.2	1.0	1.0	0.61
0	25	38.8	1.0	1.0	0.6
0	35	37.3	1.0	1.0	2.9
0.05	25	17.0	1.0	1.0	150.7
		2.6	0.35	0.06	
		19.9	0.65	0.94	3.3
0.10	25	10.8	1.0	1.0	478.9
		1.2	0.49	0.08	
		7.7	0.14	0.15	
		15.4	0.37	0.77	1.6
0.10	15	15.2	1.0	1.0	403.6
		2.5	0.47	0.10	
		19.7	0.53	0.90	2.5
0.10	35	7.6	1.0	1.0	383.3
		0.3	0.72	0.08	
		6.1	0.13	0.27	
		11.7	0.15	0.65	4.1

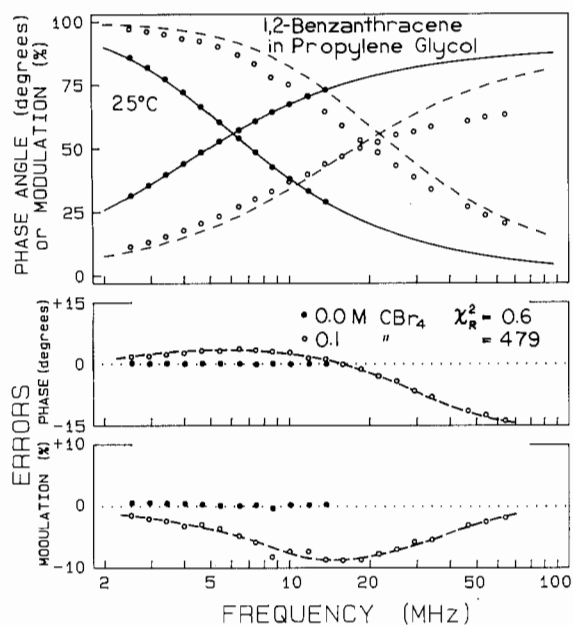


Fig. 1. Effect of quenching on the frequency-response of 1,2-benzanthracene. Data are shown for BA in the absence of CBr_4 (\bullet) and in the presence of 0.1 M CBr_4 (\circ), 25°C. The solid and dashed lines are the best single exponential fits to the data. The lower panels show the deviations between the data and the best single exponential fits in the absence (\bullet) and presence (\circ) of CBr_4 .

single photon counting [7].

The intensity decays became more complex when quenched by CBr_4 . One example is shown in fig. 1, for 0.1 M CBr_4 at 25°C. The data cannot be fit even approximately by a single exponential model (---). The mismatch is particularly evident in the lower panels, which shows the deviations between the best single decay time fit and the data. Additional single and multi-exponential analyses are summarized in table 1. In each case, the values of χ_R^2 increase several hundredfold in the presence of CBr_4 , which indicates the decays are more complex than a single exponential. The emission spectra of BA were not altered by the CBr_4 , and the results were not dependent on the duration of the measurements. Similar complex decays were observed for the quenching of indole by acrylamide and by iodide [16,20]. We interpret these effects as being the result of the time dependence of the quenching constant (eqs. (3) or (7)).

We initially attempted to fit the data using the decay law predicted by Smoluchowski, $\exp(-t/\tau$

$-2bt^{1/2})$. We were disappointed by the unreasonable values found for the interaction radii (R) and the diffusion coefficients (D), table 2. The interaction radii were invariably larger than expected, ranging from 20 to 148 Å, the diffusion coefficients were too small, and the values of χ_R^2 indicated the fit was inadequate. For example, even when the analysis yielded unreasonable values of R and D to account for the data, the values of χ_R^2 were elevated about 30-fold over the expected value. With our approximate 40 degrees of freedom a 30-fold increase in χ_R^2 means that the probability of eq. (4) being the correct model is essentially zero. A similar trend was noticed for acrylamide quenching of indole in water or propylene glycol [20]. We consistently noticed that the deviations from $\exp(-t/\tau - 2bt^{1/2})$ became more pronounced at the higher quencher concentrations. Also, when fit to $\exp(-t/\tau - 2bt^{1/2})$ the apparent values of R became larger, and the apparent values of D became smaller, at the higher quencher concentrations.

We attempted to fit the data using a fixed value of 8.5 Å for the interaction radius, which is the average value found by Ware and co-workers [7] and the value expected for molecules of this size. Fixing $R=8.5$ Å resulted in about 5-fold larger values of χ_R^2 (table 2), and still greater deviations between the measured and calculated values. However, the values of D are closer to the expected values when R is fixed at 8.5 Å. One example of these attempted fits is shown in fig. 2. It is apparent that the curve calculated (---) for $\exp(-t/\tau - 2bt^{1/2})$ cannot account for the frequency response of the emission. The differences between the data and the calculated curve are shown in the lower panels. Similar results were found at the lower quencher concentration of 0.05 M CBr_4 (fig. 3), but as might be expected the deviations are somewhat smaller.

For a period of months we did not understand the origin of the poor match between theory and experiment. In fact, we thought the system could not be described using the collisional quenching model. Finally, we questioned whether time resolution was adequate to require the more complete expression (eq. (7)). The data were analyzed using the model with radiation boundary conditions. We found that the parameters R and κ were highly correlated, so one value was always held at a fixed value in the analysis.

Table 2
Quenching of 1,2-benzanthracene by CBr_4 in propylene glycol

[CBr_4] (M)	T ($^\circ\text{C}$)	$\exp(-t/\tau - 2bt^{1/2})$			Eqs. (7)–(9)			
		R (\AA)	$10^6 \times D$ (cm^2/s)	χ_R^2	R (\AA)	$10^6 D$ (cm^2/s)	κ (cm/s)	χ_R^2
0.05	25	20.2	0.103	11.6	9.1	0.596	<150>	4.5
		<8.5> ^{a)}	0.663	55.3	<8.5>	0.641	173	4.5
0.10	25	32.0	0.0198	26.4	8.5	0.616	<150>	5.5
		<8.5>	0.688	184.5	<8.5>	0.614	149	5.5
0.10	15	147.5	0.00001	30.2	7.9	0.313	<150>	4.3
		<8.5>	0.357	172.5	<8.5>	0.292	131	4.4
0.10	35	32.3	0.0298	27.0	9.1	0.823	<150>	7.0
		<8.5>	0.995	171.0	<8.5>	0.881	171	6.9

^{a)} < > indicates the parameter was fixed at this value in the analysis.

Nonetheless, only a narrow range of R and κ values could be used to fit the data. This range of acceptable values for κ illustrated in fig. 4, which shows the dependence of R , D and χ_R^2 on κ . To construct this plot we fixed κ at the value indicated on the x -axis,

and the values of R and D were floating parameters to minimize the value of χ_R^2 . As the value of κ is increased, the apparent distance decreases and the

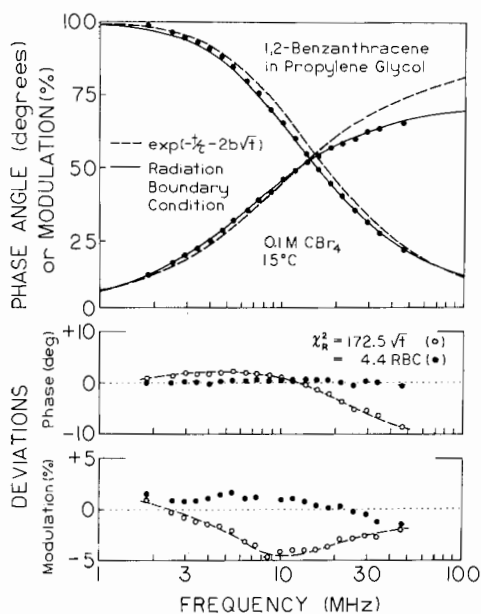


Fig. 2. Transient decay analysis of 1,2-benzanthracene quenched by 0.1 M CBr_4 , 15 $^\circ\text{C}$. The best fit of the data (\bullet) to $\exp(-t/\tau - 2bt^{1/2})$ with R fixed at 8.5 \AA is shown as a dashed line, and deviations from this fit as open circles (\circ). The best fit to eq. (7) is shown as a solid line, and the deviations as closed circles (\bullet).

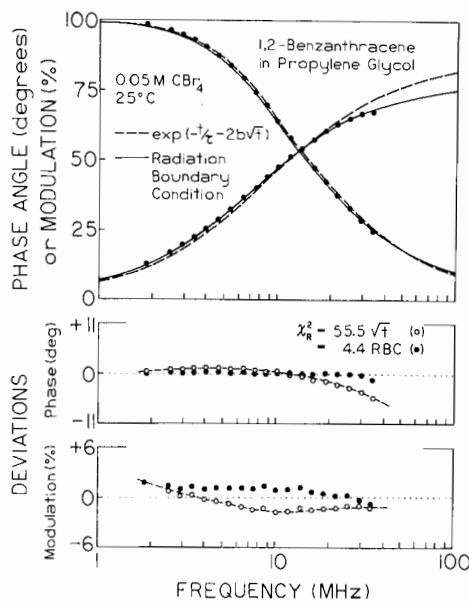


Fig. 3. Transient decay analysis of 1,2-benzanthracene quenched by 0.05 M CBr_4 , 25 $^\circ\text{C}$. The best fit of the data to (\bullet) $\exp(-t/\tau - 2bt^{1/2})$ with R fixed at 8.5 \AA is shown as a dashed line, and the deviations as open circles (\circ). The best fit to eq. (7) is shown as a solid line, and the deviations as closed circles (\bullet). For ease of comparison the deviations are shown with the same range as in fig. 2.

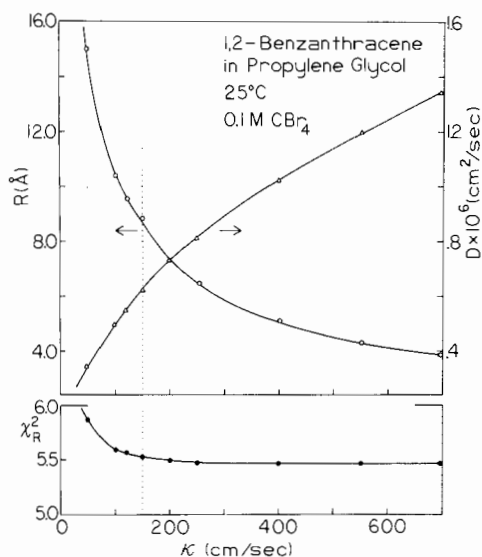


Fig. 4. Effects of κ on the values of the diffusion coefficient (D), the interaction radius (R) and the goodness-of-fit parameter (χ_R^2). The value of κ was held fixed at the value indicated on the x-axis. R and D were varied to yield the minimum value of χ_R^2 , $[\text{CBr}_4] = 0.1 \text{ M}$, 25°C .

apparent diffusion coefficient increases. The values of χ_R^2 are not very sensitive to κ , especially above values of 200 cm/s. This insensitivity is probably the result of the quenching being completely diffusion limited at the higher κ values, and hence the data are insensitive to the precise value of the rate constant for quenching. A value of $\kappa = 150$ cm/s was chosen because the interaction radii is near 8.5 \AA and the diffusion coefficient is near the expected value of $0.6 \times 10^{-6} \text{ cm}^2/\text{s}$. The most significant result of using the "radiation" model is the substantial decreases in χ_R^2 and recovery of values for R and D which are reasonable with χ_R^2 near its minimum value. For example, using either a fixed value of $R = 8.5 \text{ \AA}$ or a fixed value of $\kappa = 150$ cm/s, we obtained diffusion coefficients in good agreement with those expected for the molecular size and viscosity of this system and found previously [7,21]. The improved fit between theory and data is also evident from figs. 2 and 3. The curves calculated from eq. (7) (—) are in good agreement with the data, and the deviations are randomly distributed (figs. 2 and 3). Additionally, the temperature-dependent diffusion coefficients show an activation energy near 11 kcal/mole, in agreement with the temperature-dependent viscosity of propyl-

ene glycol, which is near 11 kcal/mole [21,22]. We conclude that our data support the complete expression for transient effects in quenching (eq. (7)) over the original expression due to Smoluchowski (eq. (4)).

The value of κ is of interest because it indicates the rate of quenching. The units of κ (cm/s) can be converted to the more familiar units for a bimolecular rate constant ($\text{M}^{-1} \text{s}^{-1}$) by calculating $k_0 = \kappa 4\pi R^2 N'$. Using $R = 8.5 \text{ \AA}$ one finds a quenching rate constant of $8.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$. Since κ is not known for other systems, it is difficult to interpret the present value. Using eq. (2) the bimolecular quenching constant (k_0) is expected to be near $0.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$, indicating that quenching is efficient and occurs as soon as the fluorophore and quencher diffuse to the encounter distance.

It should be noted that it would be difficult to detect the need for the radiation model from steady state measurements. This can be seen from using the values of k_0 and k_D derived from our experiments. According to Noyes [23,24] the observed steady state quenching constant is given by

$$k_{\text{obs}} = k_D k_0 / (k_D + k_0) . \quad (15)$$

Using our values for k_0 and k_D , one finds that the observed quenching constant is $0.38 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$, which is nearly equal to k_D . It is unlikely that this 5% difference would be noticed in a steady state experiment.

It is also of interest to compare the specific rate constant for quenching with that calculated from the kinetic theory of gases (k_c). For different reactants this rate can be calculated by

$$k_c = 2(2\pi R_g T / \mu)^{1/2} R^2 N' , \quad (16)$$

where R_g is the gas constant and μ is the reduced molecular weight [25], $\mu = M_1 M_2 / (M_1 + M_2)$ where M_1 and M_2 are the molecular weights of the fluorophore and the quencher. Using an interaction radius of 8.5 \AA and 25° one obtains $2.96 \times 10^{11} \text{ M}^{-1} \text{s}^{-1}$. Hence, the rate constant for quenching appears to be about 36-fold less than that expected for molecules in the gas phase. The alternative expression used by Noyes [23] yields a value of $1.33 \times 10^{11} \text{ M}^{-1} \text{s}^{-1}$, which is 16-fold less than the observed value. It is possible that our measurements do not yield precise estimates of k_0 because k_0 is much greater than k_D ,

Table 3
Quenching of 1,2-benzanthracene by CBr_4 in propylene glycol,
25°C

[CBr_4] (M)	Frequency range (MHz)	R (Å)	$10^6 D$ (cm^2/s)	χ_R^2
0.05	1.8–34	20.2	0.103	11.6
	1.8–25	16.9	0.163	5.5
	1.8–16	14.1	0.245	4.0
	1.8–12	12.1	0.313	3.4
	1.8–8.5	9.9	0.467	3.3
	1.8–7.4	8.0	0.65	2.6
0.10	2.5–64	32.0	0.019	26.4
	2.5–29	19.6	0.098	7.3
	2.5–25	17.5	0.134	3.2
	2.5–14	14.4	0.215	1.9
	2.5–10	11.2	0.360	0.77
	2.5–7.4	9.2	0.506	0.56
	2.5–6.4	7.9	0.644	0.45

and hence the precise values of k_0 do not have a strong influence on the data.

We questioned to origin of our ability to detect deviations from eq. (4), as compared with the time-resolved data which were found to agree with this expression [6,7]. We reasoned that our time resolution was superior to that available in the earlier measurements. To mimic a loss of time resolution we progressively deleted the data obtained at the higher modulation frequencies (table 3). As the frequency range was compressed the data converged towards the values found using eq. (7). However, a substantial reduction in the highest frequency from 64 to 8 MHz was necessary to obtain the expected values. This indicates that our resolution with a upper frequency of 8 MHz is comparable to that obtained by Ware and co-workers [6,7].

Isenberg and co-workers also examined this system using time-correlated photon counting [26]. They noted that the data could be fit to eq. (4), but the values of the derived parameters differed 3-fold from those found by Ware and co-workers [6,7]. The present results suggest the reason for this discrepancy. It seems probable that the later experiments [26] were of somewhat higher resolution, resulting in the deviations from the expected values.

6. Discussion

Our results demonstrate that the frequency-domain method provides good resolution of the complex decay laws which result from the transient effects in quenching. In particular, the data and analyses demonstrate that the radiation boundary condition model [3,4] is superior to the more familiar $t^{1/2}$ model for describing collisional quenching of fluorescence. These and similar data should be valuable for comparison of the theories for diffusion-controlled reactions with the experimental data.

Recently it has become possible to perform frequency-domain measurements to an upper frequency limit of 2 GHz [14]. These measurements allow transient effects to be seen in non-viscous solutions and for decay times near 4 ns [16,20]. Such effects are barely detectable using time-resolved measurements, even when using a picosecond laser source [27]. Using the frequency-domain measurements it should be possible to develop models and to perform measurements to recover the diffusion rates of quenchers within biological macromolecules. Such data are of interest with regards to understanding the dynamics of macromolecules [28], and for a more detailed interpretation of the results for quenching of protein fluorescence by oxygen, acrylamide and other quenchers [29–34].

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