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Laboratories and Demonstrations

Synthesis, Characterization, and Luminescence Properties of Anthrylpolyamines: An Experiment for an Integrated, Advanced Laboratory Course

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A 10-week, open-ended experiment for a junior/senior-level integrated laboratory course is described. The project involves the synthesis and instrumental characterization of two monosubstituted and two disubstituted anthrylpolyamines of varying lengths, as well as a detailed investigation of their photophysical and photochemical properties in the presence of polyanions of biological interest. Depending on the nature of the polyanion, emission quenching of the anthracene chromophore occurs by a template-directed excimer formation, or by an energy-transfer process. A correlation between the charge of the protonated anthrylpolyamines and the degree of emission quenching is also investigated. This project is ideally

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suited for introducing students to different quenching mechanisms within the context of a research-oriented, integrated laboratory experience.

Introduction

This multiweek research-oriented laboratory project was designed for use in an advanced integrated laboratory course at Haverford College. Students taking this course had either already taken or were concurrently taking a physical chemistry course (with a separate laboratory experience). The course was team-taught by an organic chemist and a physical chemist. The project, centered around the Ph.D. thesis of one of the authors [1], spanned 10 weeks and introduced students to various techniques of organic synthesis, modern instrumental characterization, and photochemical studies. It was designed to be an open-ended experiment in which students could experience the interconnected nature of the various subdisciplines of chemistry as they were applied to a research-level project. The students worked in pairs to synthesize the hydrochloride salts of two monosubstituted and two disubstituted anthrylpolyamines by two different synthetic routes. These products, as well as some of the starting materials, were then analyzed by GC–MS, FTIR, UV–vis, ^{1}H NMR, ^{13}C NMR, and ^{13}C DEPT-135 NMR spectroscopy. In the final and most interesting phase of the project, the luminescent behavior of these highly cationic anthrylpolyamines was investigated in the presence of four biological polyanions: heparin (hep), poly(L-glutamate) (PG), single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA).

This article describes the photophysical and photochemical behavior of the synthesized compounds with particular emphasis on their quenching mechanisms, topics which are usually not covered at great length in the undergraduate curriculum and for which few laboratory experiments are published. Excellent general references on fluorescence are available [2, 3].

All four of the polyanions quench the native luminescence of the anthrylpolyamines, but to different extents and by different mechanisms. Possible quenching mechanisms for the complexes of interest include excimer formation, exciplex formation, and energy-transfer quenching. Our experiments demonstrated that two of the polyanions quench the anthracene emission by anion-promoted excimer formation and the other

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two polyanions quench the emission by energy-transfer quenching. An excimer (short for **exc**ited-state d**imer**) occurs when the excited anthracene molecule aligns itself with a ground-state anthracene molecule to form an excited π-stacked dimer complex. Emission from the excimer occurs at lower energies than for the monomer, and is typically broad and structureless because emission leads to a continuum resulting from repulsion of the ground-state molecules. Energy-transfer quenching can occur when the energy of the anthracene excited state is larger than the excited-state energy of the quencher and the electronic motions of the two species are coupled. In this case, the excited-state energy of the anthracene moiety is transferred to the quencher molecule before emission can occur. The excited quencher molecule may or may not emit light as it relaxes back down to its ground state. The students were encouraged as a class to formulate possible mechanisms for the observed quenching behavior, and to design further experiments to test their hypotheses. These included obtaining a difference spectrum for the anthrylpolyamine in the presence and absence of a polyanion in order to test for the presence of excimer formation, and an enzymatic digestion experiment in order to determine whether or not the length of the polyanionic chain was important in the quenching mechanism.

Experimental

Materials and Instrumentation

Granular sodium borohydride, anhydrous sodium sulfate, 99.5% hydrogen chloride gas, 9-anthraldehyde, tetraethylenepentamine, diethylenetriamine, (3-aminopropyl)-ethylenediamine, concd HCl, NaOH, and EDTA were obtained from Aldrich; 9,10-bis(chloromethyl)anthracene was obtained from Acros; $CDCl_3$ and D_2O were obtained from Cambridge Isotope Laboratories; and heparin (sodium salt from bovine intestinal mucosa), poly(L-glutamate) (sodium salt, 15–50 kDa), single-stranded DNA (lyophilized from calf thymus), double-stranded DNA (type XV, lyophilized from calf thymus), heparinase I (lyophilized from *Flavobacterium heparinum*), and protease (Type XIV bacterial from *Streptomyces griseus*) were obtained from Sigma. The DNA contained 6.57% phosphorous by mass, according to the manufacturer's specifications.

IR spectra were recorded for 9-anthraldehyde and the monosubstituted anthrylpolyamine products as KBr pellets, and for the polyamine reactants between NaCl plates, using a Nicolet Magna 550 FTIR spectrophotometer. Proton NMR spectra were performed for the 9-anthraldehyde ($CDCl_3$) and for the anthrylpolyamine

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products (D_2O). (For spectra obtained in D_2O, the chemical shifts were standardized to the DHO peak. A Bruker 300 MHz NMR spectrometer was used to collect the spectra of the compounds. For spectra obtained in D2O, the chemical shifts were standardized to the DHO peak (Because the chemical shift of DHO is a function of its concentration, for any given anthrylpolyamine, the chemical shifts were not identical for all students).^{13}C NMR and ^{13}C DEPT-135 NMR spectra were obtained for the anthrylpolyamine products on the Bruker instrument using a drop of spectrometric grade 1,4-dioxane (VWR) as an internal standard (δ 66.50 ppm). A Hewlett–Packard 5988A mass spectrometer, coupled with a Hewlett–Packard 5890A GC, was used to record mass spectra for the anthracene-based starting materials. UV–vis spectra of the anthrylpolyamine products were obtained in aqueous solution using a Perkin Elmer Lambda 6 spectrophotometer, and luminescence measurements were performed using a SPEX Fluorolog 1681 fluorometer. The students were instructed in both the theory and the use of the modern instrumentation, and worked in pairs to obtain and to interpret the necessary spectral data.

Synthesis and Characterization

Each student team synthesized one monosubstituted and one disubstituted anthrylpolyamine. The students were given lectures outlining the general synthetic procedures, flame-drying of equipment under vacuum, anhydrous techniques, and the use of thin-layer chromatography. 1-*N*-(9-anthrylmethyl) tetraethylenepentamine (**1**) and 1′-*N*′′-(9-anthrylmethyl)-*N*,*N*′-bis-(3-aminopropyl) ethylenediamine (**2**) (Figure 1) were synthesized by analogy to the literature method [4] by stirring 0.31 g (1.5 mmol) 9-anthraldehyde with a 10-fold excess of tetraethylenepentamine or (3-aminopropyl)ethylenediamine in 25 mL chloroform. The mixture was allowed to stir for about 5 min, after which 20–25 mL activated 4-Å bead (4–8 mesh) molecular sieves were added, and the reaction was followed by TLC (6:4 hexane–ethyl acetate) until all of the original aldehyde had reacted (ca. 2 h). The molecular sieves were removed by vacuum filtration, the solution was dried (Na_2SO_4) and then concentrated in vacuo. The resulting yellow oil was dissolved in 40 mL methanol, and 0.23 g (6 mmol) sodium borohydride was added. This solution was allowed to stir overnight. The solvent was removed in vacuo and the residue was treated with 100 mL 1.5 M NaOH and then extracted with chloroform (2 × 30 mL). The combined organic extracts were subsequently extracted with 1 M HCl (2 × 75 mL). The aqueous extracts were combined, and solid NaOH was added to make the solution basic. This basic aqueous

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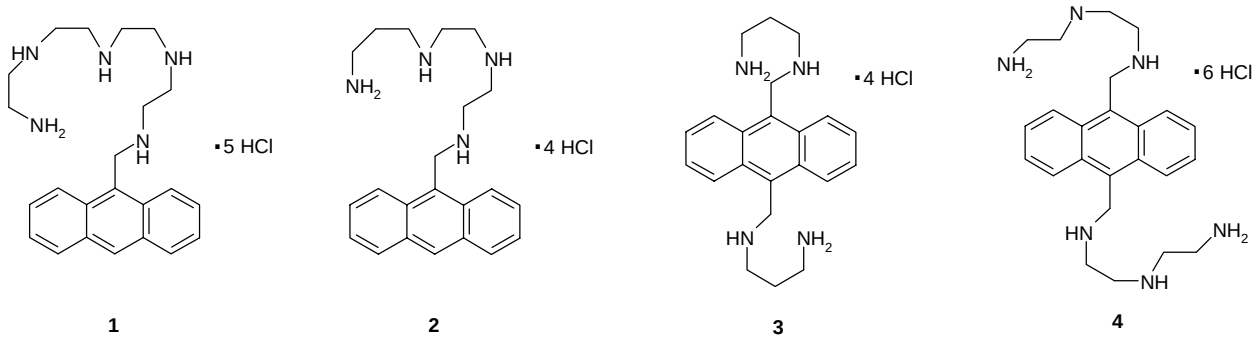

FIGURE 1. MOLECULAR STRUCTURES OF THE FOUR ANTHRYLPOLYAMINES SYNTHESIZED IN THIS EXPERIMENT.

layer was then extracted with chloroform (2 × 25 mL), and the resulting organic layer was dried (Na_2SO_4) and filtered. Hydrogen chloride gas was bubbled through the solution to precipitate the hydrochloride salts of the anthrylpolyamines. The solvent was removed in vacuo to give a pale yellow solid, which was then recrystallized from aqueous ethanol. The recrystallized product was dried overnight in a vacuum oven at ca. 95 °C. The average student yields for **1** and **2** were 18.4 ± 8.2% and 20.1 ± 10.2%, respectively.

Spectral data for **1**: UV–vis (H_2O) λ 334, 350, 367, 387 nm; emission (H_2O, 387-nm excitation) λ 417, 437, 468 nm; IR (KBr) 3436, 2958, 2673, 2429, 1594, 1478, 1097, 732 cm^{-1}; ^1H NMR (D_2O) δ 8.48 (s, 1H), 8.13 (d, *J* = 9.0 Hz, 2H), 8.02 (d, *J* = 8.4 Hz, 2H), 7.64 (t, *J* = 7.5 Hz, 2H), 7.53 (t, *J* = 7.5 Hz, 2H), 5.13 (s, 2H), 3.60 (t, *J* = 7.1 Hz, 2H), 3.34–3.43 (m, 14H) ppm; ^{13}C NMR (D_2O, dioxane) δ 130.43, 130.31, 129.91, 129.28, 127.73, 125.38, 122.41, 119.26, 44.75, 43.62, 43.49, 43.45, 43.15, 34.45 ppm; ^{13}C DEPT-135 NMR (D_2O, dioxane) δ 130.42 (CH), 129.26 (CH), 127.71 (CH), 125.36 (CH), 122.40 (CH), 44.75 (CH_2), 43.61 (CH_2), 43.48 (CH_2), 43.44 (CH_2), 43.14 (CH_2), 35.44 (CH_2) ppm.

Spectral data for **2**: UV–vis (H_2O) λ 334, 350, 367, 387 nm; emission (H_2O, 387-nm excitation) λ 416, 440, 468 nm; IR (KBr) 3401, 2955, 2912, 2761, 1607, 1439, 731 cm^{-1}; ^1H NMR (D_2O) δ 8.34 (s, 1H), 8.02 (d, *J* = 8.6 Hz, 2H), 7.91 (d, *J* = 8.4 Hz, 2H), 7.52 (dt, *J* = 7.3 Hz, *J′* = 1.3 Hz, 2H), 7.41 (dt, *J* = 7.2 Hz, *J′* = 0.6 Hz, 2H), 4.98 (s, 2H), 3.35 (t, 4H), 2.91–3.22 (m, 8H), 1.88 (pentet, 4H) ppm; ^{13}C NMR (D_2O, dioxane) δ 130.66, 130.48, 130.10, 129.46, 127.73, 125.49, 122.48, 120.06, 59.45, 45.18, 45.10, 44.53, 43.12, 42.97, 36.48, 23.78, 22.73 ppm; ^{13}C DEPT-135 NMR (D_2O, dioxane) δ

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130.50 (CH), 129.45 (CH), 127.74 (CH), 125.49 (CH), 122.48 (CH), 60.35 (CH_2), 45.16 (CH_2), 45.08 (CH_2), 44.53 (CH_2), 43.10 (CH_2), 42.98 (CH_2), 36.46 (CH_2), 23.76 (CH_2), 22.71 (CH_2).

Both 9,10-bis[(*N*-propylenediamino)methyl]anthracene (**3**) and 9,10-bis-[(1-*N*-diethylenetriamino) methyl]anthracene (**4**) (Figure 1) were synthesized according to a modification of the literature procedure [5]. Propylenediamine or diethylenetriamine (32.7 mmol) was refluxed with 0.50 g (1.8 mmol) 9,10-bis(chloromethyl)anthracene for three days under an argon atmosphere in 60 mL of toluene, which had been freshly distilled from benzophenone ketyl. After cooling, the reaction mixture was extracted with 6 M NaOH (2 × 50 mL), and then with 50 mL 1 M HCl. The aqueous acid layer was made basic with solid NaOH pellets, and extracted with chloroform (2 × 50 mL). The combined chloroform extracts were filtered through anhydrous sodium carbonate and concentrated to an oil. The hydrochloride salt was precipitated by dissolving the oil in ca. 20 mL 95% ethanol and adding 1 mL concentrated HCl. The products were recrystallized from boiling 95% ethanol–concd HCl (1:1), collected by vacuum filtration, and dried overnight in a vacuum oven at ca. 95 °C. The average student yields for **3** and **4** were 66.5 ± 7.1% and 29.4 ± 11.1%, respectively.

Spectral data for **3**: UV-vis (H_2O) λ 334, 354, 373, 393 nm; emission (H_2O, 393-nm excitation) λ 420, 445, 475 nm; IR (KBr) 3430, 2944, 2916, 2767, 2364, 1506, 1451, 759 cm^{-1}; ^1H NMR (D_2O) δ 8.37 (dd, J = 7.0 Hz, J' = 3.4 Hz, 4H), 7.75 (dd, J = 6.8 Hz, J' = 2.7 Hz, 4H), 5.25 (s, 4H), 3.34 (t, J = 8.0, 4H), 3.05 (t, J = 7.7 Hz, 4H), 2.09 (pentet, J = 6.9 Hz, 4H) ppm; ^{13}C NMR (D_2O, dioxane) δ 129.55, 127.79, 124.39, 123.93, 45.16, 42.72, 36.63, 23.81 ppm; ^{13}C DEPT-135 NMR (D_2O, dioxane) δ 127.88 (CH), 124.02 (CH), 45.25 (CH_2), 42.82 (CH_2), 36.71 (CH_2), 23.89 (CH_2) ppm.

Spectral data for **4**: UV–vis (H_2O) λ 338, 355, 373, 394 nm; emission (H_2O, 394-nm excitation) λ 421, 446, 476 nm; IR (KBr) 3425, 2961, 2727, 1586, 1459, 767 cm^{-1}; ^1H NMR (D_2O) δ 8.21 (dd, J = 6.9 Hz, J' = 3.2 Hz, 4H), 7.70 (dd, J = 6.9 Hz, J' = 3.0 Hz, 4H), 5.02 (s, 2H), 3.69 (t, J = 7.2 Hz, 4H), 3.13–3.53 (m, 12H) ppm; ^{13}C NMR (D_2O, dioxane) δ 131.76, 130.34, 126.31, 126.26, 47.04, 45.87, 45.76, 45.51, 37.78 ppm; ^{13}C DEPT-135 NMR (D_2O, dioxane) δ 130.34 (CH), 126.31 (CH), 47.04 (CH_2), 45.86 (CH_2), 45.75 (CH_2), 45.50 (CH_2), 37.77 (CH_2) ppm.

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Photophysical Studies

The four anthrylpolyamine products were titrated with four different polyanions: heparin, poly(L-glutamate), single-stranded DNA, and double-stranded DNA. Each pair of students performed only four of the 16 possible permutations of the titrations, working with the two anthrylpolyamines they had synthesized and using each polyanion in one of their four titrations. Stock solutions of the anthrylpolyamines and the polyanion titrants were prepared in pH 5.0, 0.10 M sodium acetate buffer, which contained 0.05 mM EDTA as a metal-ion complexing agent and a preservative. The concentrations of the anthrylpolyamine solutions were 6.67×10^{-5} M, the hep solution was 0.333 mM, the PG solution was 2.00 mM, and the ssDNA and dsDNA solutions were 0.200 mM. These concentrations were defined as one mole of equivalents per liter of buffer solution. An equivalent for hep was defined as a single average monosaccharide, for PG as a single amino acid residue, and for both ssDNA and dsDNA as one phosphate group. All of the stock solutions were stored in a refrigerator at 4 °C in plastic containers when they were not in use.

For each titration, the students prepared 21 plastic test tubes containing various proportions of the anthrylpolyamines and the polyanionic titrants. Automatic micropipets were used to dispense the appropriate amount of each solution to be added to the test tubes. Each tube first received 60 μL of the anthrylpolyamine stock solution. Then, an appropriate amount of polyanion was added to each test tube. The amounts of polyanion required for the titrations were defined as every 0.25 log(equivalent) units from –3.75 to +2.25 log(eq) for hep, –2.00 to +3.00 log(eq) for PG, and –3.00 to +2.00 log(eq) for ssDNA and for dsDNA. One equivalent, in this case, was defined as the number of moles of polyanion required to equal the number of moles of anthrylpolyamine added to each test tube (4.0 nmol). Finally, each tube was filled with enough buffer to bring its total volume to 4.000 mL. Thus, as an example, the test tube that contained +2.25 log(eq) PG (180 eq, 710 nmol) required the addition of 360 μL of 2.00 mM PG stock solution and 3.580 mL buffer solution to the 60 μL of 6.67×10^{-5} M anthrylpolyamine stock solution. Some of the more negative log(eq) values mandated volumes too small to be measured using the automatic pipets available; for these, the polyanion stock solutions were first diluted by a factor of 1000, and then the appropriate amounts were added to the test tubes.

After the 21 test tubes for each titration had been prepared, the fluorometer was used to measure the effects of the polyanions on the luminescence intensity of the

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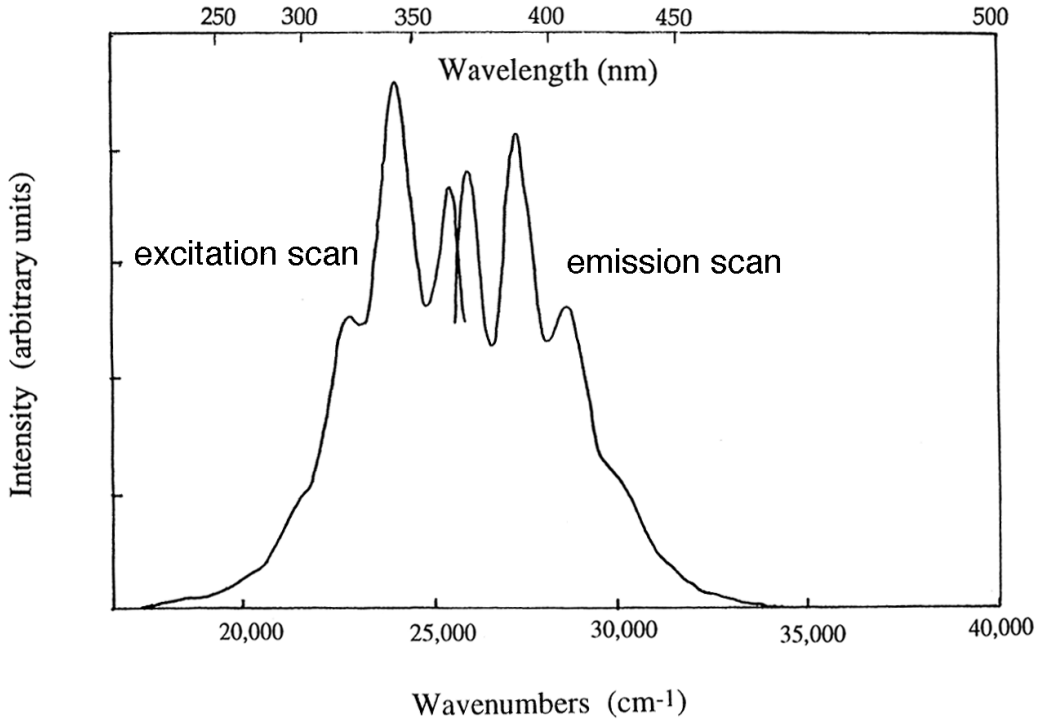

FIGURE 2. EXCITATION (λ_{em} = 416 nm) AND EMISSION SCANS (λ_{exc} = 355 nm) OF 6.67×10^{-5} M **1** IN pH 5.0, 0.10 M SODIUM ACETATE BUFFER, WHICH ALSO CONTAINED 0.05 mM EDTA.

fluorescent anthrylpolyamines. The fluorometer's excitation wavelength was set to the value of the anthrylpolyamine's lowest energy absorption in the UV–vis spectrum (typically around 390 nm), and a full emission spectrum of the anthrylpolyamine was recorded from 400 to 600 nm. Using the resulting emission maximum, a full excitation scan was also collected for each product in order to show the "mirror image" relationship between the vibrational structure of the excitation and emission spectra (Figure 2). For each of the 21 prepared solutions, the luminescent intensity was recorded at ca. 420 nm (roughly the emission maximum) for each test tube. The resulting intensities were then normalized by dividing each by the emission intensity of the test tube having the largest fluorescent intensity, and these results were graphed versus log(eq) of the polyanion used. The number of log(eq) at the minimum in the titration curve and the maximum quenching fraction (I_q/I_o) were then determined from the graph and included in a table of the class's overall titration data (Table 1).

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TABLE 1. Titration data for the emission properties (at ~ 420 nm) of the four anthrylpolyamines in the presence of four different biological polyanions. Conditions of the titrations and a description of the appbreviations used are listed in the text. Results are the average of the class's values 3–5 data points).

Compound	Polyanion Quencher	log(eq) min	I_q/I_o (at min)
1	hep	1.61 ± 0.60	0.20 ± 0.06
1	PG	2.58 ± 0.12	0.093 ± 0.017
1	ssDNA	1.75 ± 0.43	0.16 ± 0.06
1	dsDNA	2.42 ± 0.42	0.15 ± 0.05
2	hep	1.78 ± 0.00	0.25 ± 0.07
2	PG	2.75 ± 0.00	0.11 ± 0.02
2	ssDNA	2.50 ± 0.25	0.26 ± 0.18
2	dsDNA	2.38 ± 0.07	0.077 ± 0.014
3	hep	2.20 ± 0.37	0.32 ± 0.03
3	PG	3.27 ± 0.21	0.53 ± 0.09
3	ssDNA	2.33 ± 0.12	0.083 ± 0.042
3	dsDNA	250 ± 0.35	0.034 ± 0.016
4	hep	1.20 ± 1.20	0.24 ± 0.04
4	PG	2.56 ± 0.11	0.12 ± 0.02
4	ssDNA	2.50 ± 0.35	0.045 ± 0.025
4	dsDNA	2.31 ± 0.11	0.042 ± 0.006

The pooled data, which included the results of all 16 possible permutations of the four anthrylpolyamines with the four polyanions, were then analyzed by the class in a group discussion. It quickly became apparent that the hep and PG titration curves (Figure 3) were distinctly different from those of ssDNA and dsDNA for all four anthrylpolyamines. A number of possible quenching mechanisms were proposed (the students had previously received detailed lectures on luminescence spectroscopy, including discussions of electron-transfer quenching, energy-transfer quenching, and the formation of excimers and exciplexes), and the class was encouraged to design experiments which could test these hypotheses. Eventually, it was decided that a difference emission spectrum should be obtained for the test tube having the minimum

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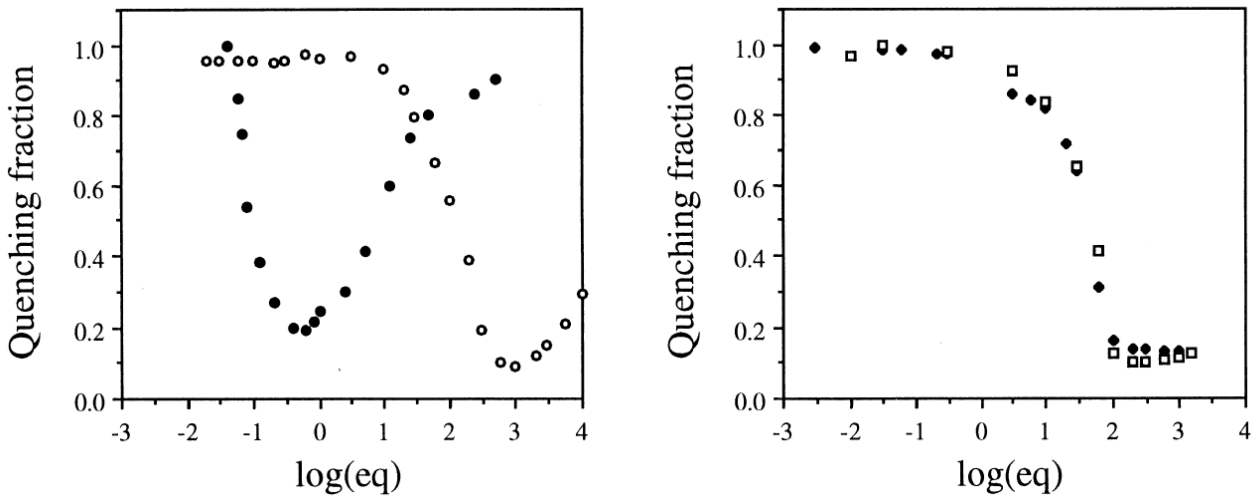

FIGURE 3. NORMALIZED TITRATION CURVES FOR 1.00 μM **1** WITH HEP (SOLID CIRCLES), PG (HOLLOW CIRCLES), ssDNA (SOLID DIAMONDS), AND dsDNA (HOLLOW SQUARES) IN pH 5.0, 0.10 M SODIUM ACETATE BUFFER, WHICH ALSO CONTAINED 0.05 mM EDTA. THE NORMALIZED EMISSION INTENSITIES WERE RECORDED AT 416 nm AFTER FOLLOWING IRRADIATION AT 390 nm. TERMS ARE DEFINED IN THE TEXT.

luminescent intensity at ca. 420 nm versus the test tube in which there was no quencher.

The purpose of this experiment was to test whether or not the positively charged anthrylpolyamines were forming excimers on the surfaces of the polyanions, leading to the observed quenching behavior. If so, the titrations would be repeated, plotting the luminescence intensities at the wavelength of the excimer emission. If there was evidence for excimer formation, a digestive enzymatic experiment could also be performed, which would break down the polyanion into smaller pieces and thereby reduce the number of possible excimers on the surface of the polyanions. Due to time constraints of the course, these experiments were not performed on all of the 16 possible titrations. Instead, only those titrations of **4** with hep and PG were investigated, and the results were generalized to the entire data set.

The difference emission spectrum for **4** with hep or PG was obtained for the test tube in the titration that had the minimum luminescence intensity at ca. 420 nm. This spectrum was normalized by dividing it by the point that had the maximum intensity. The difference spectrum was then obtained by subtracting the normalized unquenched emission spectrum of **1** from the normalized emission spectrum for the minimum in the

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titration curve. The difference spectrum represented the emission spectrum of the anthrylpolyamine excimer. The average value of the wavelength for this excimer was 488 nm. Thus, the titrations of **4** with hep and PG were repeated, recording the luminescent intensity at ca. 488 nm as a function of log(eq) of quencher.

Lastly, for the titration of **4** with hep or with PG, the test tube with the minimum luminescent intensity at ca. 420 nm was chosen and its emission intensity was recorded as a function of time, after the addition of a concentrated solution of either heparinase I or protease. The test tube was prepared with 60 μL of a 6.67×10^{-5} M solution of **4** and the appropriate amount of hep or PG, and was filled to a final volume of 3.900 mL with buffer solution. The fluorometer was set to obtain data at ca. 420 nm, integrating for 5 s every 45 s over a 2-h time period. Just before the program was initiated, 100 μL of ca. 0.25 mg mL^{-1} heparinase or protease (kept at 0 °C until needed) was added to the cuvette. Immediately after mixing, collection of the enzymatic kinetics data was begun.

Results and Discussion

The most interesting aspects of this project were the photophysical studies of the anthrylpolyamines and their emission quenching by various biological polyanions. For **1**, the excitation spectrum (λ_{em} = 416 nm) and emission spectrum (λ_{ex} = 355 nm) are overlapped on a single diagram and are depicted in Figure 2 using wavenumber units. The lowest-energy absorption and emission bands, like anthracene, display a mirror-image vibrational structure; the 0–1 bands are the most intense. The 0–0 bands for absorption and emission are shifted slightly from each other, due to the solvent rearrangements around the excited state that take place rapidly after photoexcitation, and also due to those that take place in the ground state after the fluorescence has occurred. This effect is particularly pronounced because a polar solvent (water) was used.

The highly cationic nature of anthrylpolyamines has led to the consideration of their use as luminescent probes of polyanions of biological interest [6]. Figure 3 depicts representative normalized titration curves for **1** with hep, PG, ssDNA, and dsDNA. In all of the titrations, the luminescent intensity at ca. 420 nm is initially quenched as the concentration of the polyanion is gradually increased. However, at higher concentrations of polyanion, it becomes readily apparent that the hep and PG

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polyanions quench the emission of **1** by a completely different mechanism than do ssDNA and dsDNA. The titration curves for ssDNA and dsDNA reach a minimum emission intensity that does not rebound at higher concentrations of the quencher. On the other hand, the hep and PG titration curves gradually decrease as the concentration of the polyanion is increased, until they reach a definite minimum, after which the luminescence intensity gradually begins to increase again with increasing amounts of hep or PG. Identical trends are observed for all four anthrylpolyamines, although the position and depth of the minima in their titration curves vary somewhat.

The students were encouraged to hypothesize as to why the hep and PG titrations differed from those of ssDNA and dsDNA. Possible quenching mechanisms discussed included electron-transfer quenching, intermolecular energy-transfer quenching, excimer formation, and exciplex formation. Since the anthrylpolyamines are not redox-active, the first mechanism was immediately discarded. In the case of hep and PG, two polyanions that did not contain any aromatic rings in their molecular structures, it was impossible for an energy-transfer process involving π stacking or exciplex formation to be involved in the quenching mechanism. Additionally, there would be no reason to believe that any such interaction would lead to the reversible quenching behavior observed by the presence of a well-defined minimum in these titration curves. By process of elimination, this left the possibility of excimer formation between the excited state of one anthrylpolyamine π stacked next to a second anthrylpolyamine in the ground state.

With the help of the chemical literature [6], the students were able to explain the shapes of the hep- and PG-titration curves in terms of template-directed excimer formation. At low concentrations of polyanion, the ca. 420-nm emission of the anthrylpolyamines is relatively unquenched as there are only a few interactions between the species in dilute solution. As the concentration of polyanion increases, however, the positively charged anthrylpolyamines are electrostatically attracted to the negative charges on hep or PG, which brings them in close proximity to each other on the surface of the long polyanions. This leads to the formation of some anthrylpolyamine excimers and a decrease in the emission intensity at ca. 420 nm. As the concentration of hep or PG is increased even further, the anthrylpolyamines have more polyanions to which they can bind, and because they can now spread themselves out on the surfaces of many of the polyanions, fewer of them exist in the form of excimeric pairs, and the luminescence intensity begins to increase again. Thus, it is not

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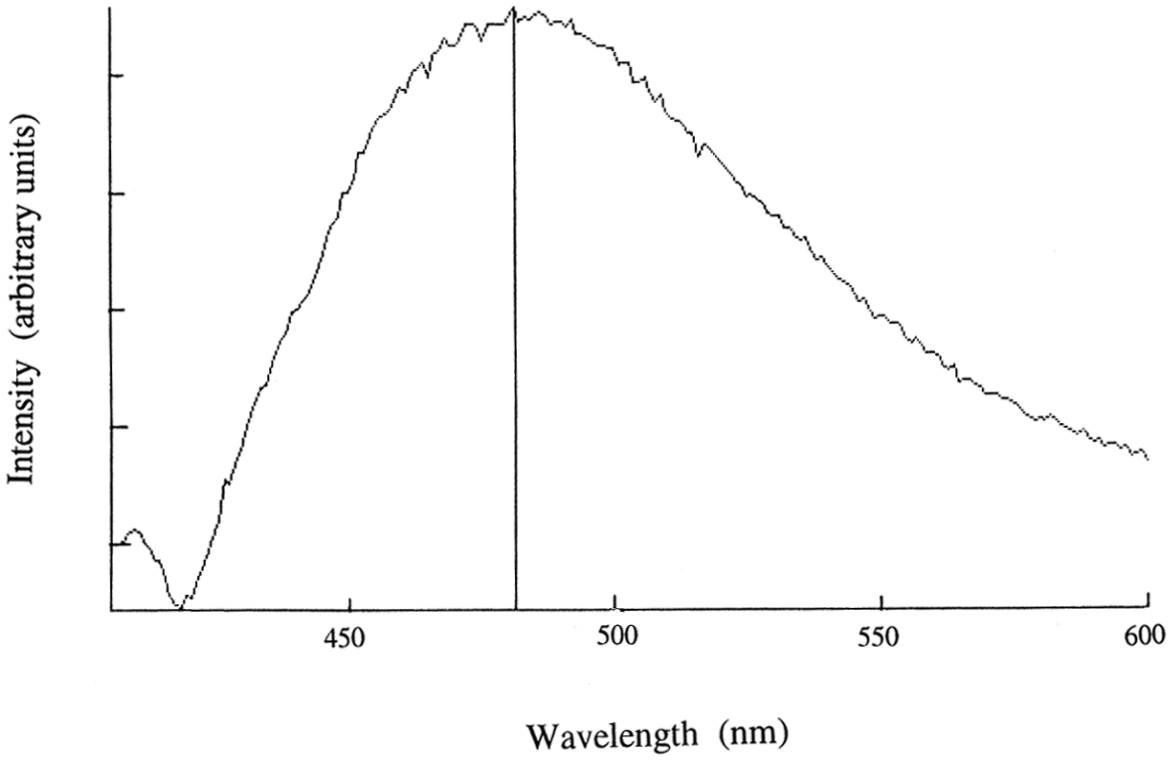

FIGURE 4. DIFFERENCE EMISSION SPECTRUM OF 1.00 μM **4** WITH 0.030 mM PG AS THE QUENCHER IN pH 5.0, 0.10 M SODIUM ACETATE BUFFER, WHICH ALSO CONTAINED 0.05 mM EDTA. THE DIFFERENCE SPECTRUM WAS OBTAINED BY SUBTRACTING THE NORMALIZED EMISSION SPECTRUM (λ_{exc} = 392 nm) OF UNQUENCHED **1** FROM THE NORMALIZED EMISSION SPECTRUM OF **1** IN THE PRESENCE OF PG. THE VERTICAL LINE AT 481 nm ILLUSTRATES THE EXCIMER'S EMISSION MAXIMUM.

the polyanion itself that quenches the emission of the anthrylpolyamines; the polyanion simply serves as an anionic template on which the anthrylpolyamines form excimers and quench each other.

This mechanism is supported by the presence of a broad, structureless emission peak at lower energy than the unquenched emission in the difference spectrum of **1** with either hep or PG. The difference spectrum was obtained by subtracting the normalized unquenched emission spectrum of **1** from the normalized emission spectrum for the minimum in the titration curve. The difference spectrum for **4** with PG as the quencher is shown in Figure 4. Of the four anthrylpolyamines, only for **2** could a difference spectrum depicting the excimer emission not be observed. As evidenced by enzymatic data supporting excimer formation, and by analogy to the other anthrylpolyamines

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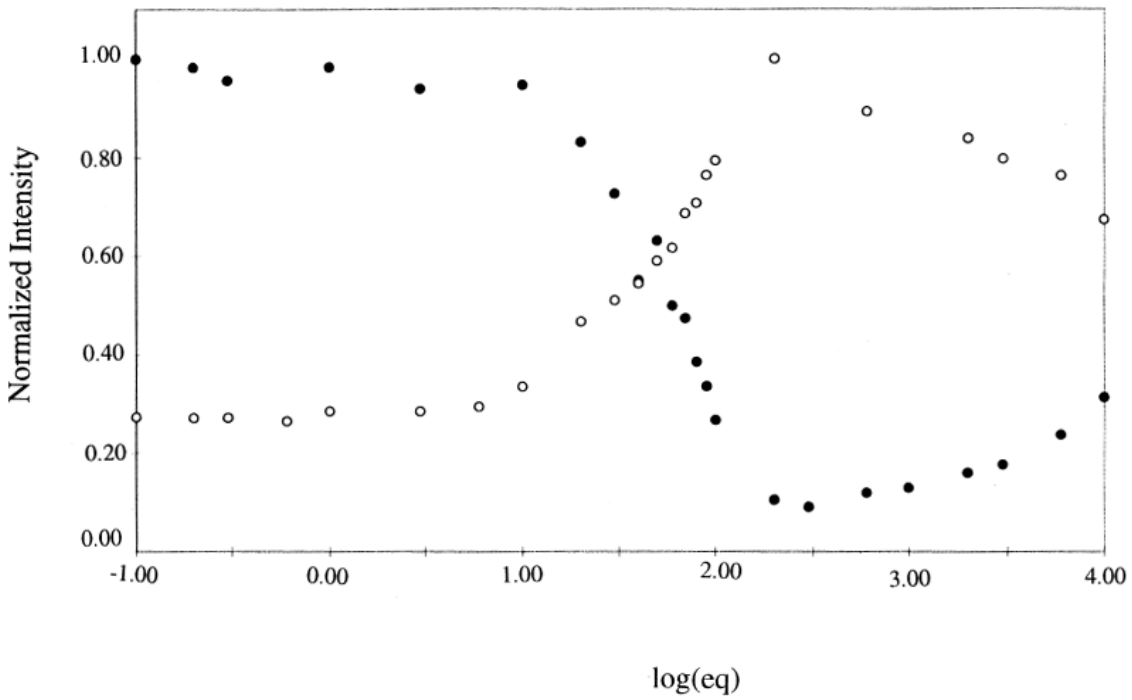

FIGURE 5. NORMALIZED TITRATION CURVE FOR 1.00 μM **4** WITH 0.030 mM PG AS THE QUENCHER IN pH 5.0, 0.10 M SODIUM ACETATE BUFFER, WHICH ALSO CONTAINED 0.05 mM EDTA. THE NORMALIZED EMISSION INTENSITIES WERE RECORDED AT 421 nm (SOLID CIRCLES) AND 500 nm (HOLLOW CIRCLES) FOLLOWING IRRADIATION AT 392 nm.

emission observed in the difference spectra of the other three anthrylpolyamines was fairly weak compared to the native emission of the anthrylpolyamines. Therefore, it is postulated that the reason excimer emission is not observed for **2** is simply a matter of the excimer emission spectrum being buried by the more-intense unquenched emission of the anthrylpolyamine at 416 nm. The other three anthrylpolyamines all showed excimer emission at 488 ± 9 nm in their difference spectra.

The titration of these anthrylpolyamines with hep or PG was then repeated, observing the excimer emission at ca. 490 nm, rather than the native emission at ca. 420 nm. The normalized titration of **4** with PG as the quencher, monitored at both 421-nm and 500-nm emission, for example, is shown in Figure 5. In contrast to the emission intensity at 422 nm, reaches a minimum and then begins to increase again with added polyanion, the emission intensity of the excimer at 500 nm increases to a maximum and then begins to decrease with added quencher. In fact, at exactly the same point in the titration curve where the emission intensity of the native anthrylpolyamine reaches a

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minimum, the excimer emission reaches its maximum, corroborating the excimer-formation hypothesis.

As an additional test of whether excimers were the principal form of quenching for the anthrylpolyamines with hep and PG, an enzymatic digestion experiment was performed. For the test tube that showed the minimum emission intensity at ca. 420 nm, ca. 8.5 mg mL^{-1} heparinase or ca. 0.25 mg mL^{-1} protease was added to the solution (the optimal concentration for use should first be determined by the instructor), and the emission intensity at ca. 420 nm was monitored as a function of time. As shown in Figure 6 for the time-based emission scan of **4** with PG in the presence of protease, the emission intensity at 416 nm increased as a function of time as the enzyme began to digest PG into smaller and smaller pieces. As the polyanion was broken down into shorter chains, there was less chance of an excimer being able to form on the shorter pieces of the polyanions. The emission intensity of the native anthrylpolyamines therefore began to increase with time, eventually reaching a plateau that was nearly equal in intensity to an equimolar concentration of the unquenched anthrylpolyamine. Because the native emission intensity returns to almost exactly the same level as if there had never been any added polyanion, the template-directed excimer formation hypothesis used to explain the quenching of the anthrylpolyamines by hep or PG appears to be not only the principal, but also the only quenching mechanism observed in these titrations.

Since the emission intensity at ca. 420 nm does not rebound with increased quencher in the case of ssDNA or dsDNA, a different mechanism than the one proposed above for hep and PG must be involved in these systems. Unlike hep or PG, DNA contains aromatic base pairs that can π-stack with the anthrylpolyamines. This π-stacking interaction can quench the anthrylpolyamine's luminescence by one of two mechanisms: intermolecular energy transfer or the formation of an exciplex between the anthrylpolyamine and the DNA. No difference spectrum to detect exciplex formation was observed, and thus energy transfer was deemed to be the most likely of the two quenching mechanisms. Regardless of the mechanism, however, the nature of the interaction is such that the DNA is itself acting as the quencher, rather than simply directing the formation of excimers on its surface. Since DNA is also negatively charged, and could in theory direct such a template-directed interaction, one can infer that the anthrylpolyamines either π-stack more efficiently with the DNA base pairs

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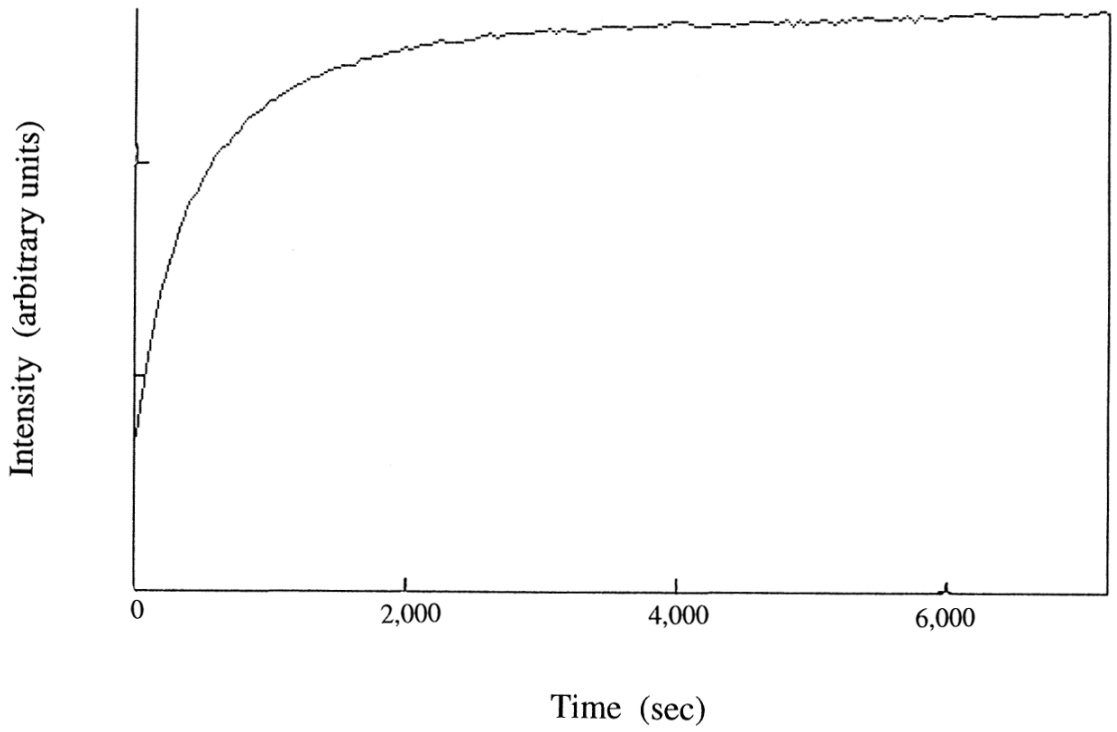

FIGURE 6. ENZYMATIC DIGESTION OF 1.00 μM **4** AND 0.030 mM PG IN pH 5.0, 0.10 M SODIUM ACETATE BUFFER, WHICH ALSO CONTAINED 0.05 mM EDTA AFTER THE ADDITION OF 100 μL OF 0.236 mg mL^{-1} PROTEASE. THE EMISSION INTENSITY WAS MONITORED AT 421 nm FOR 2 HOURS, FOLLOWING CONTINUOUS IRRADIATION AT 392 nm.

than with each other, or simply that there are statistically more base pairs to interact with than there are neighboring anthrylpolyamines.

Table 1 lists the pertinent results of all 16 of the possible permutations between the four anthrylpolyamines and the four polyanions. The $\log(eq)_{min}$ is defined as the log(eq) of quencher which was necessary to reach the minimum luminescence intensity at ca. 420 nm in the titration curves. For hep and PG, this is a well-defined value; for ssDNA and dsDNA, this is the point at which the minimum intensity first appeared. These values are related to the affinity of the anthrylpolyamines for the polyanionic quenchers. Since this interaction is primarily an electrostatic one in the case of hep and PG, one would expect that **4** (which has a +6 charge) would have a smaller value of $\log(eq)_{min}$ than **1** (+5 charge) and **2** or **3** (+4 charges). This is indeed the observed pattern for these anthrylpolyamines. It is also predicted that hep, which has on the average more than one negative charge per equivalent, should have a smaller value of

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$\log(eq)_{min}$ than the other three quenchers, which all have a single negative charge per equivalent. The data also support this hypothesis. Since the interaction of the anthrylpolyamines with ssDNA and dsDNA is more complicated because they can have π-stacking interactions in addition to an electrostatic attraction, the differences between the affinities of the four polyanions for DNA are less predictable.

The maximum quenching fraction, I_q/I_o, which indicates how efficient the quenching is at this minimum in the titration curves, is also listed in Table 1 for each anthrylpolyamine–quencher combination. The smaller the value of I_q/I_o, the greater the quenching effect. No definitive trends among the four different polyamines could be identified from the data. However, in all but two cases, the value of I_q/I_o was smaller for ssDNA and dsDNA than for hep and PG. This would indicate that the ability of the π-stacked anthrylpolyamine–base-pair interaction is more efficient at quenching the emission intensity than is the formation of excimers between two anthrylpolyamines. In all cases, the dsDNA was slightly more efficient at quenching the anthrylpolyamine's luminescence than was the ssDNA. In both cases, following an initial electrostatic attraction, the anthrylpolyamines can π stack with the nucleotide bases of DNA. The increased quenching efficiency of dsDNA versus ssDNA might therefore be explained by intercalation of the planar anthrylpolyamines into the dsDNA double helix. In order to test whether or not intercalation is actually occurring in dsDNA, polarized emission studies and DNA melting experiments could be performed on these systems.

Conclusions

This 10-week advanced laboratory experiment introduced junior and senior chemistry majors and biochemistry students to two alternate methods of organic synthesis, several different laboratory techniques, the use of modern instrumentation, spectral analysis, emission spectroscopy, the design of experiments to test their own hypotheses, and communication of their research results in both oral and written form. It also integrated several important areas of chemistry together in a single project, as well as encouraged the students to delve deeply into a research-oriented laboratory experience. The majority of students taking this course reacted positively to the common theme of an open-ended research project, the intellectual freedom from cookbook-type experimentation, and the critical thinking skills necessary to interpret the results.

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