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Fluorescence quenching of peptidyl tryptophan by external quenchers

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FLUORESCENCE QUENCHING OF PEPTIDYL TRYPTOPHAN BY EXTERNAL QUENCHERS

by

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ABSTRACT

Fluorescence quenching data have been collected on several small peptides (1-4 residues) which contain tryptophan. These data have been analyzed by a variety of methods. The classical Stern-Volmer equation is utilized to obtain Stern-Volmer plots of fluorescence quenching data. Substantial upward curvature is observed in the Stern-Volmer plots of all the peptides studied.

Data were also analyzed via the modified Stern-Volmer static quenching model. Although this model could explain the deviation from simple Stern-Volmer behavior found in SV plots, the parameters used by the model were often physically "unrealistic": Therefore, another form of data analysis was used. The newly proposed Peak dynamic quenching model was tested. This model could explain the deviation from simple Stern-Volmer behavior in a physically tangible manner. A value for quenching efficiency (ε) is derived from the Peak model.

Finally, charge effects and ionic strength effects on fluorescence quenching are investigated. Some very interesting ionic strength effects have been found in the quenching data of several peptides. In the case of $^{+}$TGG^{-} vs. acrylamide, there appears to be a cation effect on quenching.

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Of the twenty amino acids present in proteins, only two exhibit fluorescence: tryptophan and tyrosine. Each contains an aromatic function; however, the magnitude of tryptophan's fluorescence is much greater than that of tyrosine. As a result, when fluorescence measurements are made on peptides, the majority of any fluorescence emission can be credited to the tryptophan residue(s).

As protein structure becomes complex, a tryptophan residue in the protein may be on the exterior of the structure or buried within the structure. Thus, tryptophan residues in a protein can reside in various microenvironments.

The fluorescence of tryptophan can be "quenched" by other molecules called quenchers. In the case of tryptophan, fluorescence is often quenched when the quencher has physical contact with the indole ring. Thus, the degree of quenching which occurs can be used as an indicator of how exposed a trytophan residue is in a protein structure.[REF] This information could thus be used to determine detailed information about a potential structure for the protein. This is one reason why fluorescence quenching studies are carried out on trytophan containing polypeptides.

The study of quenching of the fluorescence of trytophan-containing peptides (2-4 residues) was the primary objective of this research project. The microenviromental condition around a tryptophan residue was manipulated, and the effects of these manipulations on quenching were analyzed. These manipulations included variable positioning of positive and negative

charges or near the tryptophan residue, the prescence or absence of ionic strength control in solution, and the use of charged and uncharged quencher molecules.

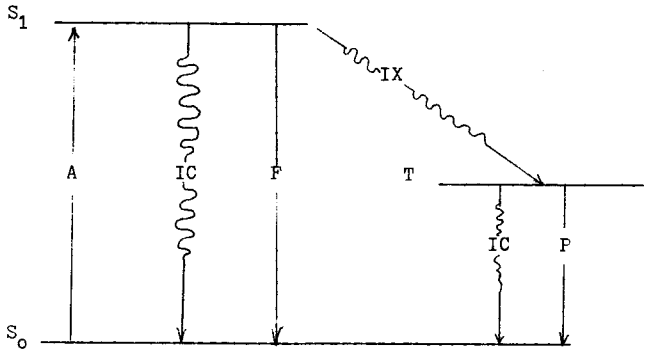
Fluorescence data were analyzed using several fluorescence quenching mechanisms. Experimantal data were plotted according to the classical Stern-Volmer equation [REF#], the modified Stern-Volmer equation for static quenching [REF1,7], and fit to the newly proposed Peak mechanism.[REF3] Of the three mechanisms utilized, experimental results correlated best with the Peak mechanism of dyanmic quenching.

Essential to the understanding of the results obtained from this research project is a fundamental knowledge of the three aforementioned fluorescence quenching mechanisms as well as the electronic transitions which occur when a molecule is subjected to radiation. The latter can best be studied with the aid of a Jablonski diagram. (Figure 1).[REF 6]

Before electronic excitation , molecules are in their "ground" electronic state, S_0. When a molecule is exposed to radiation, a photon of the appropriate energy can be absorbed causing electrons to be promoted to a higher electronic energy orbital, and the molecule is said to be in an excited state, S_1. These electronic states can be singlet (S) or triplet (T) states. Once a molecule is in an excited electronic state, it can return to the more energetically favorable ground state by several pathways. The molecule can release energy by fluorescenc (F), intersystem crossing (IX), internal conversion (IC), or phosphorescence (P). The above electronic transitions can be expressed in the following manner.

Figure 1

Jablonski Diagram



S_o = ground electronic state (singlet)
S_1 = excited electronic state (singlet)
A = absorbance
F = fluorescence
P = phosphorescence
T = triplet electronic state
IC = internal conversion
IX = intersystem crossing

$$M + h\nu \xrightarrow{k_e} M^* \quad \text{(absorbance)} \qquad (1)$$

$$M^* \xrightarrow{k_f} M + h\nu \quad \text{(fluorescence)} \qquad (2)$$

$$M^* \xrightarrow{k_{nr}} M + \text{heat} \quad \text{(internal conversion or intersystem crossing)} \qquad (3)$$

Upon introducing a quencher molecule into the system, another form of deactivation of the excited state can occur. It can be expressed as follows; REF 5

$$M^* + Q \xrightarrow{k_q} M + Q \quad \text{(quenching)} \qquad (4)$$

If a molecule is exposed to steady illumination, the rate of formation of the excited state molecules can be written as:

$$\frac{d[M^*]}{dt} = k_e - (k_f + k_q[A] + k_{nr})[M^*]. \qquad (5)$$

Using the steady state assumption for $[D^*]$ yields

$$k_e = (k_f = k_q[A] + k_{nr})[D^*]. \qquad (6)$$

Given that the quatum yield (Φ) for emission from $[D^*]$ in the absence of $[Q]$ is:

$$\Phi = \frac{k_f[D^*]}{k_e} = \frac{k_f}{k_f + k_{nr}} \qquad (7)$$

and in the prescence of Q is:

$$\Phi_A = \frac{k_f[D^*]}{k_e} = \frac{k_f}{k_f + k_q[Q] + k_{nr}} \qquad (8)$$

then the following ratio can be written:

$$\frac{\Phi_o}{\Phi_A} = \frac{k_f + k_q[Q] + k_{nr}}{k_f + k_{nr}} = 1 + \frac{k_q}{k_f + k_{nr}}[Q]. \qquad (9)$$

This result is the Stern-Volmer equation. It also appears in the following form:

$$\frac{\Phi_o}{\Phi_A} = 1 + k_q \tau_o [Q] = 1 + K_{sv}[Q] \qquad (10)$$

where τ_o is the fluorescence lifetime in the absence of quencher and K_{sv} is called the collisional quenching constant. REF 5 Therefore, a plot of $\frac{\Phi_o}{\Phi_A}$ versus $[Q]$ will yield a straight line with slope K_{sv} and intercept of 1. The lifetime (τ_o) of fluorescence can be measured, and hence, from a derived value of slope (K_{sv}), the second order rate constant (k_q) can be calculated.

For experiments carried out during this project, plots of F_o/F versus $[Q]$ were drawn. This was acceptable because the ratios $\frac{\Phi_o}{\Phi_A}$ and $\frac{F_o}{F}$ were identical under the experimental conditions utilized. All data collected were graphed according to the Stern-Volmer equation. However, instead of the plots being linear, a certain degree of upward curvature was consistently observed. As a result of this deviation from theoretical predications, another quenching mechanism, the modified Stern-Volmer equation including static quenching, was applied.

This mechanism for quenching considers the possibility that a quencher molecule and fluorophore molecule may be very close to each other at the time of fluorophore excitation, and thus, an instantaneous quench occurs. This type of quench is called a static quench. It follows that as quencher concentration increases,

the probability of a "static" quench also increases.[REF 7] The modified Stern-Volmer equation is derived using the following model. (See Figure 2).

Any quencher which enters the volume V will cause an immediate static quench of the fluorophore when it is excited. The probability that an M will have a Q within V must be calculated. If the system is loaded with molecules of Q in N increments, the probability that M will not have a Q in V after the first increment would be $\left(1 - \frac{V[Q]}{N}\right)$. If survival of M after the first increment does not affect the survival of M after the second increment, then the probability that M does not have a Q in V becomes $\left(1 - \frac{V[Q]}{N}\right)\left(1 - \frac{V[Q]}{N}\right)$. Hence, after N increments, this probability becomes $\left(1 - \frac{V[Q]}{N}\right)^N$. As N approaches infinity, the following equation results:

$$P_\infty = e^{-V[Q]} \qquad (11)$$

This equation expresses the probability of M still not having a Q in V.[REF]

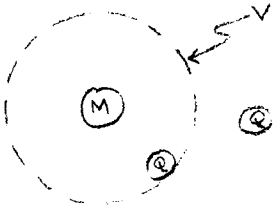
Utilizing steady state assumptions again, the following "modified" Stern-Volmer equation is obtained.[REF 1,7]

$$\frac{F_o}{F \cdot e^{V[Q]}} = \left(1 + k_q \tau_o [Q]\right). \qquad (12)$$

Plots of $F_o/F e^{V[Q]}$ versus $[Q]$ for various values of V are made until a linear plot is obtained. The slope of this line is K_{sv}, and from this value, a second order rate constant for quenching can be derived.

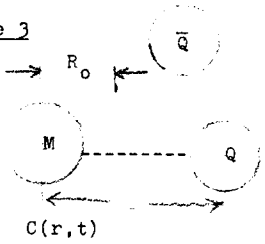
Figure 2

Static Quenching Model



where M is the fluorophore, [Q] is the quencher, and V is the static quenching volume.

Figure 3



M = fluorophore
Q = quencher (pair)
$\bar{Q}$ = quencher (competitive)
r = pair separation distance
R_o = pair contact radius

Note: $r \leq R_o$ for quench to occur

Peak Dynamic Quenching Model

Thus, the upward curvature observed in Stern-Volmer plots can be explained. However, this mechanism does possess a flaw. The static quenching volume appears to be much larger than the fluorophore-quencher contact volume. This suggests a quench without physical contact or even close proximity between the fluorophore and quencher. Because of this seemingly physically unrealistic characteristic of the modified Stern-Volmer mechanism, another quenching mechanism was sought.

The Peak mechanism of dynamic quenching was the final mechanism investigated. The actual mathematical derivation of the Peak equation is quite complex and very lengthy.[REF 3] Thus, it will suffice to briefly describe the Peak model and how it is used to analyze fluorescence data. The Peak mechanism is based on the following model. (Figure 3).

The expression $\Im(r,t)$ represents the pair concentration (fluorophore-quencher pairs) in solution as a function of separation diatance (r) and time (t). This model is a dynamic model, and a fluorophore can be quenched by its "conjugate" quencher molecule or a "competitive" quencher molecule diffusing in for collision. In order for quenching to occur, the separation distance of a pair must be less than or equal to the pair's contact radius (R_o). Thus, physical contact is necessary for quenching.

Another deviation of the Peak nechanism form classical ideas on fluorescence quenching is that the Peak mechanism proposes that k_e and k_q are dependent on quencher concentration. (See equations 1 and 4). Using these assumptions, a computer program

based on the Peak mechanism was created. When fed fluorescence data and molecular parameters, such as fluorophore-quencher contact radii, lifetime and diffusivity, the program, using a non-linear least squares fitting procedure, computes the best "fit" of the data to one parameter. From this fit, another parameter called the quenching efficiency can be derived which may have a value of 0 to 1. It indicates the probability that a quench will occur upon fluorophore-quencher collision.

During this research project, fluorescence quenching data were collected on several peptides containing a tryptophan residue using iodide and acrylamide as quenchers.[5] A relative quantum yield (ϕ_R) determination was taken on each peptide using N-acetyl-L-tryptophanamide (NATA) as a reference. The ϕ_R measurements were used to compare fluorescence efficiencies of various peptides and to check on the "freshness" of peptides used in experiments by comparing new values of ϕ_R against older values.

Experimental results were also used to provide fluorescence quenching data to test the Peak mechanism. Using the Peak fitting program, quenching efficiencies could be generated for several of the peptides tested.

Finally, in some of the experiments, results pertaining to charge effects and ionic strength effects on quenching were obtained. Iodide and acrylamide were the quenchers used in all quenching experiments; the former carries a negative charge while the latter is a neutral species. All peptides selected for study contained a tryptophan residue and 1-3 glycine residues. The peptides tested were in zwitterion form, resulting in a manipul-

ation of positive and negative charge on or around the tryptophan residue. Iodide quencher also bears a negative charge, and thus, a "charge" effect on quenching could be studied. Charge effects were examined further by including ionic strength control in solution. Some very interesting charge effects were observed for both iodide and acrylamide quenching experiments with ionic strength control.

Experimental

I. Instrumentation

The following is a description of all equipment and instrumentation used during this research project.

To record absorbance measurements on peptide solutions, a Beckman DU Spectrophotometer was utilized. A hydrogen lamp served as a radiation source, and the monochromator slit width was set at 0.70 mm.

To obtain fluorescence emission data on various peptides, a Hitachi Perkin-Elmer Model MPF-2A Spectrofluorometer was used. A xenon lamp was used as a radiation source. Excitation radiation used in the experiments ranged from 280 to 295 nm.

In the preparation of the phosphate buffer solution, an Orion Research Model 701/digital Ionalyzer was used to measure the pH of the solution. For the majority of pipetting, SMI digital micropettors were used.

Finally, for data analysis, an Apple Computer was employed to process the experimental data and to print Stern-Volmer plots of fluorescence data. Most Stern-Volmer plots were saved on storage diskettes.

II. Chemicals

The peptides and peptide derivatives used during this research project obtained from Sigma Chemical Company were N-Acetyl-L-Tryptophanamide (NATA), L-Tryptophyl-Glycine (TG), and Glycyl-L-Tryptophan (GT). Acquired from Vega Biochemicals were L-Tryptophyl-Glycyl-Glycine (TGG), Glycyl-L-Tryptophyl-Glycyl-Glycine (GTGG), Glycyl-L-Tryptophyl-Glycine (GTG), and Glycyl-Glycyl-L-Tryptophane (GGT).

Other chemicals used for experimental purposes were potassium iodide (99+%) and sodium phosphate dibasic heptahydrate obtained from Aldrich Chemical Company, and acrylamide obtained from Sigma Chemical Company.

Chemicals supplied by the Union College Chemical stockroom were potassium chloride, sodium chloride, and sodium thiosulfate. Each of these compounds was high purity grade.

III. Solution Preparation

A. Buffer Solution

In order to keep test solutions at a pH of approximately 6, so that peptides would be in zwitterion form, a 0.05M phosphate buffer was used. The buffer solution is prepared by first adding 1.34 grams of Na_2HPO_4 to 100ml. of triply distilled deionized water (3XDD water), and then adding concentrated HCl dropwise until a pH of approximately 6 is achieved. A 0.05M H_2PO_4/HPO_4^{2-} buffer solution results.

B. Ionic Strength Adjuster

For the purpose of ionic strength control in some of the solutions tested, an ionic strength adjuster (ISA) solution was prepared from either KCl or NaCl. This solution was used to keep an observed peptide in a constant "ionic environment". Preparation of each type of ISA solution was accomplished as follows. For a 5.0M NaCl solution, approximately 2.92g of NaCl was added to a 10.0ml volumetric flask and diluted to the mark with 3XDD water. A 2.5M KCl solution was prepared by adding 18.64g of KCl to a 100ml volumetric flask and diluting to the mark with 3XDD water.

C. Iodide Quencher Solution

During the course of experimentation pertaining to iodide quenching, various methods were utilized to prepare a KI stock solution of appropriate molarity which would cover a given quencher concentration range during a quenching experiment. At the earliest stages of testing, it was necessary to prepare two solutions of KI. One (Quencher A) solution had a concentration of 2.5M and the other (Quencher B) solution had a concentration of 1.0M. Two separate solutions were needed in order to cover the desired range of quencher concentration because of pipetting equipment restrictions. These stock solutions would be prepared in the following manner. To prepare a solution of 2.5M KI, 41.5g of KI were added to a 100ml volumetric flask. In order to prevent formation of I_3^-, some $Na_2S_2O_3$ (0.0025g) was also added to this flask. Dilution to the mark with 3XDD water resulted in a solution 2.5M in KI and 1.0×10^{-4}M in $Na_2S_2O_3$. The 1.0M KI quencher solution was prepared in the following way. A 20ml volume of 2.5M KI quencher solution was pipetted into a 50.0ml volumetric flask. An additional .001g of $Na_2S_2O_3$ was added to the flask, and the flask was filled to the mark with 3XDD water. Both of these solutions were stored in brown bottles in the dark to further insure that no I_3^- would form.

When micropettors of various delivery ranges became available, it was sufficient to prepare a single KI solution. The concentration of the single KI stock solution was 2.5M and its preparation is described above.

In later iodide quenching experiments, it was necessary to use a 5.0M KI stock solution. To accomplish this, 83.0g of KI and 0.0025g $Na_2S_2O_3$ were added to a 100ml volumetric flask and diluted to the mark with 3XDD water. The resulting solution was 5.0M KI and $1x10^{-4}$M $Na_2S_2O_3$. This solution was stored in a brown bottle and in the dark.

For the most recent tests, a 5.0, KI solution was used. This solution was prepared as above; however, only 10.0ml of solution were produced. To achieve this, 8.3g of KI and approximately .0003g of $Na_2S_2O_3$ was added to a 10.0ml volumetric flask and diluted to the mark with 3XDD. This solution was stored in the dark when not in use.

D. Acrylamide Quencher Solutions

1. Without ISA added.

At the outset of acrylamide quenching studies, the acrylamide solution was prepared without ionic strength control. The initial concentration of the acrylamide stock solution was 2.0M. This was prepared by adding 1.42g of acrylamide and 1.0ml of .05M PO_4^{3-} buffer to a 10.0ml volumetric flask. This was diluted to the mark with 3XDD water. The resulting solution was 2.0M in acrylamide and .005M in phosphate buffer. In subsequent experiments, the concentration of acrylamide stock solution was also made at 6.0M.

2. With Ionic Strength control.

For acrylamide quenching studies under constant ionic strength, the acrylamide quencher solution was prepared in the following manner. A 2.85g quantity of acrylamide was added to a 10.0ml volumetric flask. For ionic strength control, 2.92g of NaCl were added to this flask. After dilution to the mark with 3XDD water, a solution which was 4.0M acrylamide and 0.5M NaCl was the result.

In later experiments of this nature, KCl was used in place of NaCl as the ionic adjuster salt. In this case, 2.85g of acrylamide and .373g of KCl were added to the 10.0ml volumetric flask and then diluted to the mark with 3XDD water. The resulting solution was 4.0M acrylamide and 0.5M KCl.

E. Peptide Stock Solution

1. For ϕ_R measurements.

The peptide stock solution for relative quantum yield determinations was prepared by adding enough solid peptide to approximately 15-20ml of 3XDD water in a vial until an absorbance between .4-.5 was obtained. Only a very small amount of solid peptide was needed.

2. For Quenching measurements.

For experimental runs, the peptide stock solution was prepared by adding enough solid peptide to approximately 25ml. of 3XDD water to achieve a solution whose absorbance was .7 to .9 at λ_{ex} = 280nm. To conserve on solid peptide, later experiments used a peptide stock solution whose absorbance was .5-.7 at λ_{ex} = 280nm.

IV. Procedures

A. Measurement of Relative Quantum Yield (ϕ_R)

Before any peptide was used in experiments, its relative fluoroscence quantum yield was measured. This was accomplished by comparing a peptide's fluorescence against the fluorescence of NATA, which serves as a reference for all peptides used in these experiments. The measurement of ϕ_R for each peptide serves two purposes. First, the ϕ_R measurement permits the comparison of the magnitude of fluorescence of each peptide with that of the other peptides. Secondly, these ϕ_R values are used to check the "freshness" of each peptide by comparing newly obtained values with previously determined values.

To obtain a ϕ_R value for a peptide of interest, it is necessary to execute the following procedures. After filling two vials with approximately 15-20ml of 3XDD water, some solid NATA is placed in one vial and some solid "test" peptide is placed in the other vial. Each solution is mixed, and absorbance readings are taken on the Beckman DU at 280nm using a slit width of 0.7mm. Enough solid NATA and test peptide are added to their respective solutions until an absorbance reading of 0.4-0.5 is obtained. Using a digital pipette, 1.00ml of NATA solution is added to a 10.0ml volumetric flask, and 1.00ml of test peptide solution is added to another 10.0ml volumetric flask. Then, 1.00ml of .05 phosphate buffer (pH approximately 6.0), is added to each of the volumetric flasks. Finally, the two solutions are diluted to the mark with [illegible] water.

The Perkin Elmer MPF-2A Spectrophotometer is then placed in the following operating mode:

λ excitation = 280nm	ex.slit=5.0	Sample 3(4) Sensitivity	Med. scan speed
	em.slit=5.0	Ratio 2	Med. chart speed

Some of the NATA solution is then placed into a cuvette, and a fluorescence emission spectrum is run from 300 to 400nm. With this achieved, the cuvette is emptied, cleaned, and filled with the test peptide solution. The recording chart is wound back and fluorescence emission spectrum is run from 300 to 400nm on the test peptide solution with all instrumental settings the same as for the NATA spectrum.

The relative fluorescence quantum yield is then calculated from these data and absorbance data. The maximum height of the emission spectra peaks are measured with a ruler. ϕ_R values are derived from the following equation,

$$\phi_R = \frac{H_p}{A_{280P}} \quad \frac{A_{280NATA}}{H_{NATA}} \qquad (13)$$

where H_p = height of test peptide's maximum emission peak; H_{NATA} = height of NATA's maximum emission peak; A_{280P} = absorbance of the test peptide solution; and $A_{280NATA}$ = absorbance of NATA solution.

B. Iodide Quenching measurements with Ionic Strength Adjuster.

Two quencher ranges were covered in experiments which included ionic strength control. In earlier runs, an iodide concentration range of 0 to 0.250M was spanned; however, in later experiments, this iodide quencher range was expanded to cover 0 - 0.500M. For the earlier experiments, KCl was used in the ISA solution, while the later experiments utilized NaCl in the ISA solution. In order to achieve constant ionic strength in solution, it was necessary to prepare each quencher concentration in the range of interest in separate 10.0ml volumetric flasks. Depending on the number of quencher concentrations in the range, anywhere from 9 to 13 solutions had to be prepared for each "run". Each test consisted of three "runs".

The solutions needed for these tests were 0.05M Phosphate buffer (pH 6.0), peptide solution (A $\sim$ .7 - .9), 2.5M KCl (ISA), 5.0M NaCl (ISA), 2.5M KI (Quencher), 1.0M KI (Quencher) and 5.0M KI (Quencher). Two SMI micropettors were also essential for solution preparation. Solutions containing the proper quencher concentration ranges were prepared according to the following two tables. All solutions were diluted to the mark with 3XDD water.

TABLE 1

Protocol for I^- Quenching at Constant Ionic Strength

Q Concentration Range = 0 - .250M KI

Sol#	Quencher A (2.5M) KI	Quencher B (1.0M) KI	ISA (KCl 2.5M)	Buffer	Peptide	[Q]
1	-	-	1.00ml	1.0ml	1.0ml	0
2	-	0.300ml	0.880ml	1.0ml	1.0ml	0.030
3	-	0.450ml	0.820ml	1.0ml	1.0ml	0.045
4	-	0.600ml	0.760ml	1.0ml	1.0ml	0.060
5	-	0.750ml	0.700ml	1.0ml	1.0ml	0.075
6	0.400ml	-	0.600ml	1.0ml	1.0ml	0.100
7	0.550ml	-	0.450ml	1.0ml	1.0ml	0.138
8	0.700ml	-	0.300ml	1.0ml	1.0ml	0.175
9	0.850ml	-	0.150ml	1.0ml	1.0ml	0.212
10	1.00ml	-	0	1.0ml	1.0ml	0.250

It should be noted that in some experimental tests, solutions having quencher concentrations of .0125M, .019M, .0225M, and .038M were also prepared. They were made using a similar type of protocol as above. Also, in later experiments, when more digital pipettes were obtained, it was only necessary to prepare Quencher type stock solution of 5.0M KI.

TABLE 2

Protocol for I^- Quenching at Constant Ionic Strength

Quencher Concentration Range = 0 - 0.500M KI

Sol#	Quencher (5.0M KI)	ISA (5.0M NaCl)	Buffer	Peptide	[Q]
1	0	1.00ml	1.0ml	1.0ml	0
2	0.100ml	0.900ml	1.0ml	1.0ml	0.050
3	0.350ml	0.650ml	1.0ml	1.0ml	0.175
4	0.500ml	0.500ml	1.0ml	1.0ml	0.250
5	0.600ml	0.400ml	1.0ml	1.0ml	0.300
6	0.700ml	0.300ml	1.0ml	1.0ml	0.350
7	0.800ml	0.200ml	1.0ml	1.0ml	0.400
8	0.900ml	0.100ml	1.0ml	1.0ml	0.450
9	1.00ml	0	1.0ml	1.0ml	0.500

Once the solutions were prepared, the actual fluorescence measurements could be made using the procedure described below.

Some of solution #1 (no quencher present) was placed into a clean fluorescence cuvette. After the cuvette was seated in the cell compartment of the PE MPF-2A Spectrofluorometer, the excitation shutter and PMT shutter were opened. By adjusting the sample sensitivity and emission slit width, a recorder deflection of approximately 90% was obtained. The excitation slit width was almost always kept at 5. With signal optimization, the chart paper was advanced slightly. Now actual test run measurements could be made. Using the "spike" method described below, five repeat fluorescence measurements were taken on a solution of a given quencher concentration.

This method of measurement was devised in order to reduce the amount of time a solution would be exposed to excitation radiation, and thus reduce the chance of any photodecomposition from occurring. The method is executed as follows. With the chart recorder in servo mode, the excitation shutter is opened first and the PMT shutter is opened immediately afterwards. After a few seconds, when the recorder pen has stabilized at maximum deflection, the excitation shutter is closed first and then the PMT shutter. The chart paper is advanced slightly and the whole procedure is repeated. Five measurements are taken on each test solution.

When all measurements were finished on a given solution, the sample cuvette was removed, emptied, cleaned, and then filled with the next solution to be tested. This procedure was repeated until all of the solutions in a "run" were measured. Three runs were carried out on each peptide tested.

The following table lists the instrumental settings for all peptides measured in this manner.

TABLE 3

Instrumental Settings for I^- Quenching Studies (with ISA)

Peptide	λ ex		λ em		ex slit		em slit		sample sensitivity		ratio record	
	*A	**B	A	B	A	B	A	B	A	B	A	B
NATA	280	290	354	354	5	5	5	5	3	3	2	2
$^+$GGT$^-$	280	290	354	354	5	5	5	5	4	4	3	2
$^+$GTG$^-$	290	295	354	354	3	5	8	5	4	4	3	2
$^+$GTGG$^-$	280	290	354	354	5	5	5	5	4	4	3	2
$^+$GT$^-$	280	290	354	354	3	5	8	5	4	4	3	2
$^+$TG$^-$	280	290	354	354	5	5	5	5	3	3(4)	3	2
$^+$TGG$^-$	---	290	---	354	-	5	-	6	-	4(5)	-	2

*A = [Q] range of 0 - .250M
**B = [Q] range of 0 - .500M

C. Iodide Quenching Measurements without Ionic Strength Adjuster

To gain some insight concerning ionic strength effects and charge effects on quenching, some iodide quenching experiments were carried out in the absence of ionic strength adjuster. The stock solutions needed for this work were a peptide solution (A ~.5 - .7), buffer solution (.05M, pH~ 6), and a 5.0M KI solution (quencher). The procedure used in these experiments is described below.

The first solution to be made was the "cuvette" sample solution. To a clean 10.0ml volumetric flask were added 1.0ml of peptide stock solution and 1.0ml of buffer solution. The solution was then diluted to the mark with 3XDD water.

Using a glass pepette, 3.0ml of the "cuvette" sample was placed into a fluorescence cuvette which was then seated in the PE MPF-2A sample compartment. The excitation shutter and PMT shutter were opened, and the sample was exposed to the radiation source. Sample sensitivity and emission slit width were adjusted until a deflection of approximately 90% was obtained on the recorder. After these adjustments were made, five repeat fluorescence measurements were taken on the sample using the "spike" method.

The PMT shutter and the excitation shutter were closed, the sample compartment was opened, and an aliquot (30 ul) of quencher solution (5.0M KI) was added to the cuvette. The sample solution was stirred with the tip of the micropettor which was wiped clean

after each stir. The sample compartment was closed, the excitation and PMT shutter were opened, and five more repeat measurements were taken on the sample solution with quencher using the "spike" method. This procedure was repeated until enough quencher aliquots were added to the cuvette to span the desired quencher concentration range.

The peptides tested in this fashion and their respective instrumental settings are listed in the following table.

TABLE 4

Peptide	λ_{ex}	λ_{em}	ex slit	em slit	sample sensitivity	ratio
NATA	290nm	354nm	5	7	3	2
$^{+}$TGG^{-}	290	354	5	4	4	2
$^{+}$GGT^{-}	290	354	5	6	3	2

(Note: $^{+}$TGG^{-} and $^{+}$GGT^{-} were chosen because they showed greater affinity to charge effects.)

D. Acrylamide Quenching Measurements with Ionic Strength Control

This type of quenching experiment was undertaken to see the effects of ionic strength on the quenching of the fluorescence of certain peptides. The stock solutions needed for these experiments were 5.0M NaCl solution, acrylamide solution (4.0M acryl, 0.5M NaCl), peptide solution (A ~ .5 - .7), and .05M phosphate buffer solution (pH ~ 6).

The preparation of a "cuvette" solution was carried out as follows. A 10.0ml volumetric flask was cleaned and 1.0ml of peptide solution, 1.0ml of buffer solution, and 1.0ml of 5.0M NaCl solution were placed in it. The solution was then diluted to the mark with 3XDD water thus producing a starting solution containing 0.005M phosphate buffer and 0.50M NaCl.

Next, 3.0ml of "cuvette" solution was pipetted into a fluorescence cuvette. This cuvette was then seated in the sample compartment of the PE MPF-2A. After closing the sample compartment, the excitation and PMT shutter were opened and sample sensitivity and emission slit were adjusted to achieve a recorder deflection of 90%. Once this was accomplished, the chart paper was advanced, and five more measurements were taken on the sample. The excitation shutter and PMT shutter were then closed, and the sample compartment was opened. An aliquot of acrylamide solution was micropetted into the cuvette, and the solution was stirred with the tip of the micropetter. The micropettor was wiped clean after each stir. Since the added acrylamide solution contained 0.5M NaCl, the ionic strength was unchanged by the quencher additive.

These quencher solution additions were repeated until the desired quencher concentration range had been covered. The peptides tested and their respective instrumental settings are listed in the following table.

TABLE 5

Peptide	λ_{ex}	λ_{em}	ex slit	em slit	sample sensitivity	ratio
NATA	295	354	5	8	4	2
$^{+}$TGG^{-}	295	354	5	8	4	2
$^{+}$GGT^{-}	295	354	5	9	4	2

Similar acrylamide quenching experiments were carried out using KCl as an ionic strength adjuster. The procedure for these tests was the same as above except the quencher solution was 4.0M acrylamide and 0.5M KCl, and the composition of the "cuvette" sample solution was 1.0ml peptide solution (A ~ .5 - .7), 1.0ml buffer solution, 2.0ml of 2.5M KCl solution, and 6.0ml of 3XDD water. The peptides used and their respective instrumental settings were as follows.

TABLE 6

Peptide	λ_{ex}	λ_{em}	ex slit	em slit	sample sensitivity	ratio
$^{+}$TGG^{-}	295	354	5	9	3	2
$^{+}$TG^{-}	295	354	5	7	4	2

E. Acrylamide Quenching Measurements without Ionic Strength Control

This type of quenching experiment was carried out on several peptides and over two concentration ranges. The first set of experiments spanned the quenching concentration range of 0 - .212M acrylamide. The second group covered the quencher concentration range of 0 - .293M acrylamide. In each case, the solutions required for the tests were a peptide stock solution (A ~ .5 - .7), buffer solution (.05M phosphate), and a quencher solution (acryl.). An SMI micropettor was used to deliver microliter aliquots of quencher solution. The procedure used to obtain fluorescence data was as follows.

The "cuvette" solution was prepared by placing 1.0ml of peptide solution and 1.0ml of buffer solution into a 10.0ml volumetric flask and then diluting to the mark with 3XDD water. From this flask, 3.0ml of solution was pipetted into a fluorescence cuvette, and the cuvette was seated in the sample compartment of the PE MPF-2A. The sample compartment was closed, the excitation shutter and PMT shutter were opened, and adjustment of the sample sensitivity and emission slit produced a maximum recorder deflection of 90%. The excitation and PMT shutters were then closed and the chart paper was advanced. Five measurements were taken on the solution using the "spike" method.

After these measurements, the sample compartment was opened and an aliquot of quencher solution was added to the cuvette. The

tip of the micropettor was used to stir the solution, and it was wiped clean after each use. The sample compartment was closed, and five fluorescence measurements were taken on the solution. This procedure was repeated until the desired quencher concentration range was covered. The peptides tested in this fashion and their respective instrumental settings are listed in the following table.

TABLE 7

Peptide	λ_{ex}		λ_{em}		ex slit		em slit		sample sensitivity		ratio	
	A	B	A	B	A	B	A	B	A	B	A	B
NATA	295	295	354	354	5	5	5	5	3	3	2	2
$^{+}$GGT^{-}	295	295	354	354	5	5	6	8	4	4	2	2
$^{+}$GTG^{-}	295	295	354	354	5	5	6	8	4	4	2	2
$^{+}$GTGG^{-}	295	295	354	354	5	5	6	9	4	4	2	2
$^{+}$TG^{-}	295	295	354	354	5	5	5	8	4	4	2	2
$^{+}$GT^{-}	295	295	354	354	5	5	6	10	4	4	2	2
$^{+}$TGG^{-}	-	295	-	354	-	5	-	8	-	4(5)	-	2

NOTE: A = quencher conc. range 0 - .212M acrylamide
B = quencher conc. range 0 - .293M acrylamide

F. Selection of Excitation and Emission Wavelength for Fluorescence Measurements

For iodide quenching experiments at lower quencher concentrations, an excitation wavelength of 280 nm was chosen. At this wavelength, the tryptophan residue absorbed quite well, and competitive absorption by the I^- ion was insignificant at the concentrations used.

However, for the higher quencher concentration range for iodide quenching, an excitation wavelength of 290 nm was used. Absorbance by the tryptophan residue was good at this wavelength, and competitive absorbance by the I^- was again insignificant. At the higher quencher range, absorption by I^- at 280 nm was not insignificant.

For acrylamide quenching studies, an excitation wavelength of 295 nm was selected. Absorbance by the tryptophan residue was satisfactory, and competitive absorbance by the acrylamide could be corrected in a data analysis program. (see Data Analysis).

An emission wavelength of 354 nm was selected after running several fluorescence emission spectra on the peptides tested. It was found that maximum fluorescence emission occurred at this wavelength.

G. Data Analysis

To analyze fluorescence quenching data, several computer programs were written by Professor Thomas C. Werner to be used with an Apple Computer. Also, some commercial software was utilized to plot experimental data. The programs written by Professor Werner were called SV CALC3, SV CALC4, and SV CALC AVE. The commercial software used were Epson Plot and Sciplot. A brief explanation of each of the programs will be given.

SV CALC3

This program handled iodide quenching data obtained by the multivolumetric method. The inputs required for this program were quencher concentration and height of fluorescence emission peak (mm) at that concentration. The program took the data and calculated F_0/F values at each concentration and stored these figures in a data file. Later these figures could be retrieved from storage and be processed into Stern-Volmer plots.

SV CALC4

This program was used to process quenching data obtained by the "cuvette" method. All acrylamide quenching data and some iodide quenching data were analyzed by this program. The values needed for input were initial cuvette solution volume, volume of quencher used per addition, molarity of quencher solution, and the number of quencher additions per run. With this data, the program computed F_0/F values and their respective quencher concentrations. Essential to this program was the subroutine that corrected for dilution effects and, in the case of acrylamide quenching, for competitive absorption effects. Both of these corrections will be discussed below.

As quencher solution is added to the sample solution, the volume of the system increases. This causes the fluorescence intensity to decrease. This can be seen by studying the following equation.

$$F = kC = \frac{kn}{V} \quad \text{where } n = \text{const.} \quad (14)$$

It is apparent that as volume (V) increases, fluorescence (F) decreases.

The program adjusts for this by using the following mathematical expression.

F_c = intensity corrected for dilution
F_u = intensity uncorrected for dilution
V_i = initial volume (3.0ml)
V_t = total volume = $(V_i + nV_q)$

where n = # of quencher additions
V_q = Vol. of quencher addition.

$$F_c = \frac{k\,n}{V_i} \quad (15) \qquad \text{and} \qquad F_u = \frac{k\,n}{V_t} \quad (16)$$

Equating these expressions yields

$$F_c V_i = F_u V_t$$

or

$$F_c = \frac{F_u(V_i + nV_q)}{V_i}$$

Thus, using this equation, the program derives a corrected fluorescence value.

At an excitation wavelength of 295 nm, the molar absorptivity of acrylamide is .23. According to Parker (ref 8) the fluorescence intensity is reduced by the factor 10^{-Dd}, where D = Absorbance/cm and d= effective pathlength (cm/2).

Thus $$10^{-Dd} = 10^{-A/2} \qquad (17)$$

and $$F_c = \frac{F_u}{10^{-A/2}} = F_u \times 10^{A/2} \qquad (18)$$

In the case of acrylamide quenching, the correction of raw fluorescence values for dilution and competitive absorption effects is made using the following equation in the program.

$$F_c = F_u \times \frac{(V_i + nV_q)}{V_i} \times 10^{A/2} \qquad (19)$$

In the case of iodide quenching, only dilution effect is corrected for using the equation which follows.

$$F_c = F_u \times \frac{(V_i + nV_q)}{V_i} \qquad (20)$$

SV CALC AVE

This program was created to calculate the average F_0/F value and its standard deviation at a given quencher concentration. It was used for acrylamide and iodide data analysis.

Epson Plot & Sciplot

These programs were purchased commercial software that were used to create plots of experimental data.

Data Storage

Stern-Volmer plots of experimental data were stored on floppy diskettes.

Results

A. Relative Quantum Yield (ϕ_R) Determinations

TABLE 8

Peptide	Experimental Values	Avg. Exp. Value	Literature Value **
NATA *	1.00	1.00	1.00
$^{+}GGT^{-}$	0.34, 0.33	0.34	0.37
$^{+}GTG^{-}$	0.32	0.32	0.34
$^{+}GTGG^{-}$	0.30, 0.30, 0.30	0.30	0.32
$^{+}GT^{-}$	0.19, 0.24, 0.20 0.25, 0.25 (new)	0.21 0.25	0.30
$^{+}TG^{-}$	0.70, 0.71, 0.73 0.72, 0.68 (new)	0.71 0.70	0.62
$^{+}TGG^{-}$	0.46, 0.45, 0.46 0.46, 0.45, 0.46 (new)	0.46 0.46	--
$^{+}LTL^{-}$ ***	0.51, 0.48, 0.49	0.49	--

* NATA was used as a reference and thus was given a value of 1.00.

** Literature values were obtained from reference 2 .

(new) refers to peptides that were purchased during the project.

*** $^{+}LTL^{-}$ was not used in quenching experiments.

B. Fluorescence Quenching Data: Peptides vs. Iodide (w NaCl)

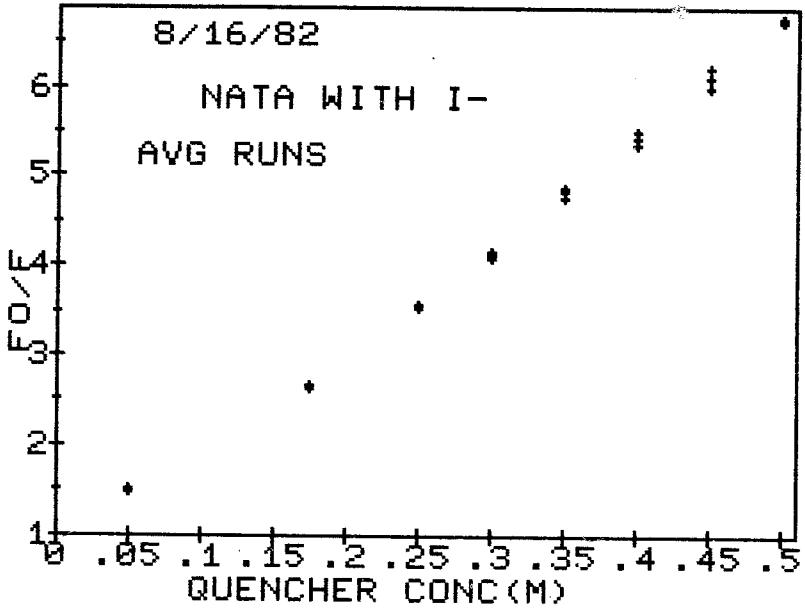
Table 9

	Average F_o/F Values and Std. Dev.													
$[I^-]$	NATA		$^+GGT^-$		$^+GTG^-$		$^+GTGG^-$		$^+GT^-$		$^+TG^-$		$^+TGG^-$	
0	1	-	1	-	1	-	1	-	1	-	1	-	1	-
.050	1.49	.01	1.12	.01	1.18	.01	1.16	.01	1.11	.01	1.39	.01	1.31	.01
.175	2.65	.02	1.48	.01	1.68	.01	1.65	.01	1.47	.01	2.46	.03	2.09	.02
.250	3.53	.02	1.71	.01	2.03	.02	1.98	.02	1.72	.01	3.24	.04	2.63	.06
.300	4.12	.02	1.88	.01	2.27	.01	2.21	.01	1.90	.01	3.77	.06	2.99	.06
.350	4.81	.06	2.06	.01	2.52	.01	2.49	.05	2.06	.02	4.29	.03	3.41	.05
.400	5.43	.08	2.22	.01	2.74	.01	2.69	.06	2.23	.01	4.96	.10	3.79	.02
.450	6.10	.10	2.37	.03	3.00	.01	2.95	.03	2.40	.01	5.80	.08	4.19	.09
.500	6.76	.02	2.54	.01	3.25	.02	3.17	.06	2.56	.02	6.33	.06	4.54	.07
# runs*	3		3		2		3		3		3		3	

* These are the number of experimental runs that were Averaged

This is the data which was collected in quenching experiments on peptides vs. iodide over the quencher concentration range of 0-.500M I^- and in the presence of 0.500M NaCl (ISA). The figure on the following page illustrates how quenching data is presented in a Stern-Volmer plot. Stern-Volmer plots of the remaining peptides can be found in the Appendix. The slight upward curvature present in the classical Stern-Volmer (SV) plot should be noted.

Figure 4


8/16/82
NATA WITH I-
AVG RUNS
FO/F
1
2
3
4
5
6
0 .05 .1 .15 .2 .25 .3 .35 .4 .45 .5
QUENCHER CONC(M)

Fluorescence Quenching Data: Peptides vs. Acrylamide (w/o ISA)

Table 10

Average F_o/F Values and Std. Dev.

[Acryl]	$^+$GGT$^-$		$^+$GTG$^-$		$^+$GTGG$^-$		$^+$GT$^-$		$^+$TG$^-$		$^+$TGG$^-$	
0	1	-	1	-	1	-	1	-	1	-	1	-
.039	1.34	.01	1.34	.01	1.29	.01	1.33	.01	1.57	.01	1.45	.01
.078	1.71	.01	1.74	.01	1.63	.01	1.65	.01	2.19	.02	1.92	.02
.116	2.14	.03	2.12	.01	2.00	.02	2.06	.01	2.88	.05	2.41	.03
.154	2.60	.03	2.58	.04	2.42	.04	2.44	.01	3.67	.07	2.91	.01
.192	3.03	.04	3.02	.05	2.86	.07	2.90	.01	4.60	.08	3.43	.06
.229	3.57	.05	3.50	.07	3.34	.06	3.36	.01	5.50	.08	4.00	.08
.265	4.08	.06	4.03	.07	3.78	.10	4.00	.02	6.52	.09	4.63	.08
.301	4.62	.05	4.58	.08	4.34	.06	4.51	.05	7.53	.13	5.29	.08
# runs*	3		3		5		3		5		4	

* These are the number of experimental runs whose data was used to compute the average.

This is the data obtained from quenching experiments on peptides vs. acrylamide over the quencher concentration range of 0-.301M acrylamide and without ionic strength control. The figure on the following page shows how this data is presented on a Stern-Volmer (SV) plot. SV plots of the remaining peptides can be found in the Appendix. Once again the upward curvature in the SV plot must be noted. Data for NATA vs. acrylamide is given below.

Table 11

[Acryl]	0		.063		.126		.187		.247		.306	
AVG F_o/F	1	-	2.32	.01	4.01	.05	5.98	.05	8.26	.02	11.00	.01

Figure 5

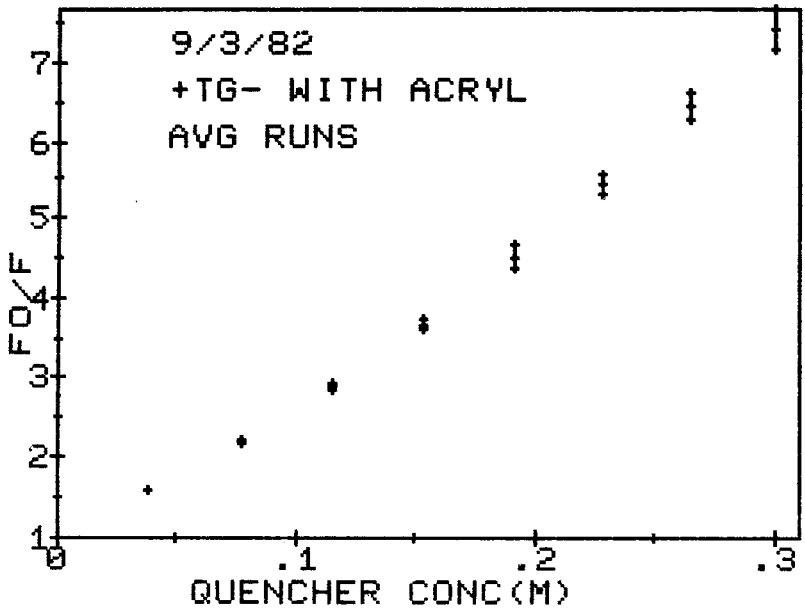


Table 12

Peptides vs. Iodide
(Lower [Q] Range)
Average F_o/F^* Values and Std. Dev.

$[I^-]$	NATA		$^+GTGG^-$		$^+GGT^-$		$^+GT^-$		$^+TG^-$		$^+GTG^-$	
0	1	--	1	--	1	--	1	--	1	--	1	---
.0125	1.12	.01	1.00	.01	1.01	.01	1.01	.01	1.07	.01	----	--
.019	1.18	.02	1.03	.02	1.04	.01	1.02	.01	1.11	.01	----	--
.025	1.25	.01	1.06	.01	1.06	.01	1.05	.01	1.17	.02	----	--
.030	----	--	1.06	.01	1.07	.01	----	--	1.20	.01	----	--
.038	1.36	.02	1.11	.01	1.10	.01	1.08	.01	1.30	.03	1.15	.01
.045	----	--	1.13	.01	1.12	.01	----	--	1.33	.01	----	--
.050	1.52	.03	1.17	.01	----	--	1.15	.01	1.43	.03	1.22	.02
.060	----	--	1.20	.01	1.18	.01	----	---	1.47	.01	----	--
.075	1.76	.02	1.27	.01	1.22	.01	1.19	.01	1.61	.03	1.31	.01
.100	2.02	.01	1.37	.01	1.31	.01	1.26	.02	1.89	.03	1.40	.01
.138	2.51	.02	1.56	.01	1.43	.01	1.42	.04	2.30	.02	1.59	.05
.175	3.00	.07	1.76	.01	1.57	.01	1.52	.02	2.74	.02	1.73	.02
.212	3.51	.08	1.96	.01	1.72	.02	1.67	.03	3.20	.03	1.93	.04
.250	4.10	.04	2.19	.01	1.86	.02	1.81	.05	3.68	.01	2.09	.03
.275	----	--	----	--	----	--	----	--	----	--	2.22	.02
.300	----	--	----	--	----	--	----	--	----	--	2.33	.02

* These are the average values of ALL runs carried out at the 0-.250M Quencer concentration range. They are average values of 3-6 runs.

The Stern-Volmer plots of these data are located in the Appendix.

Table 13

Peptides vs. Acrylamide
(Lower [Q] Range)
Average F_0/F Values* and Std. Dev.

[Acryl]	NATA		$^{+}TG^{-}$		$^{+}GTGG^{-}$		$^{+}GGT^{-}$		$^{+}GT^{-}$		$^{+}GTG^{-}$	
0	1	---	1	---	1	---	1	---	1	---	1	---
.019	1.35	.01	1.28	.02	1.14	.01	1.16	.01	1.16	.01	1.17	.01
.033	1.74	.02	1.57	.02	1.29	.01	1.35	.01	1.33	.01	1.34	.01
.057	2.14	.02	1.87	.02	1.43	.02	1.52	.01	1.50	.01	1.52	.03
.076	2.58	.02	2.18	.02	1.57	.03	1.71	.01	1.67	.01	1.70	.03
.094	3.01	.02	2.51	.01	1.71	.02	1.91	.02	1.85	.02	1.90	.03
.112	3.48	.03	2.74	.05	1.89	.03	2.12	.02	2.04	.01	2.10	.02
.129	3.96	.04	3.18	.03	2.03	.03	2.31	.03	2.22	.02	2.30	.04
.146	4.43	.04	3.47	.03	2.21	.03	2.51	.03	2.43	.01	2.48	.02
.163	4.95	.03	3.77	.06	2.36	.04	2.73	.04	2.65	.04	2.71	.01
.180	5.51	.03	4.23	.03	2.55	.05	2.92	.04	2.88	.03	2.93	.03
.196	6.07	.02	4.55	.05	2.76	.08	3.17	.06	3.10	.06	3.15	.02
.212	6.70	.03	4.97	.01	2.97	.04	3.43	.03	3.32	.05	3.37	.04

* Avg runs computed for three runs
** w/o 1.6

The results listed above are the data obtained from quenching experiments run at lower Q concentrations. The plots of this data can be found in the Appendix.

C. Fluorescence Quenching Data: Analyzed by the Modified Stern-Volmer Static Quench Model

Table 14

Peptide	Iodide (w ISA) *		Acrylamide (w/o ISA) **	
	K_{sv}	V	K_{sv}	V
NATA	8.36	.55	17.6 ± .2	1.75 ± .04
$^{+}TG^{-}$	6.66 ± .16	.75 ± .04	12.31 ± .28	1.58 ± .07
$^{+}TGG^{-}$	5.68 ± .17	.35 ± .05	9.73 ± .23	0.97 ± .07
$^{+}GT^{-}$	2.30 ± .16	.36 ± .08	5.82 ± .33	1.65 ± .13
$^{+}GTG^{-}$	3.39 ± .15	.38 ± .07	----	----
$^{+}GTGG^{-}$	3.16 ± .19	.43 ± .08	6.10 ± .19	1.42 ± .08
$^{+}GGT^{-}$	2.37 ± .13	.31 ± .07	7.45 ± .19	1.18 ± .06

* Data covering quencher conc. range of 0-.500M I^{-} used in fitting program.
** Data covering quencher conc. range of 0-.301M acryl. used in fitting program.

The parameters above were generated from a non-linear least squares fit program which was written by Professor David Peak. The program selected various V values, and using quenching data as input, would fit the data with the best straight line. From this line a value of K_{sv} could be derived. The modified Stern-Volmer equation is the basis of these results.

$$\frac{F_0}{F\, e^{V[Q]}} = 1 + K_{sv}[Q] \qquad (21)$$

D. Fluorescence Quenching Data: Analysis via the Peak Dynamic Quenching Model

Table 15

Peptide	Quencher*	ISA**	ε ***
NATA	I^-	yes	.40 ± .02
NATA	A	no	.73 ± .02
NATA	A	yes	.74 ± .04
$^+TG^-$	A	no	.73 ± .02
$^+TG^-$	I^-	yes	.46 ± .02
$^+TGG^-$	A	yes	.65 ± .03
$^+TGG^-$	I^-	yes	.49 ± .04
$^+GT^-$	A	no	.87 ± .01
$^+GT^-$	I^-	yes	.38 ± .01
$^+GGT^-$	A	yes	.77 ± .03
^+GGT-	I^-	yes	.33 ± .02
$^+GTG^-$	I^-	yes	.45 ± .04
$^+GTGG^-$	I^-	yes	.45 ± .03

* Quencher: I^- = iodide ; A = acrylamide

** ISA : yes = 0.500M NaCl; no = no ISA

*** ε : This parameter is the quenching efficiecy and it is obtained from a fitting program.

Once again a non-linear least squares fitting program written by Professor David Peak is used to obtain a fit of experimental data; however, this time data is fit to the Peak dynamic quenching model and the quenching efficiency parameter is obtained.

E. Fluorescence Quenching Data: Data obtained from Ionic Strength Manipulation in Solution

The following tables contain the results of quenching experiments which incorporated the manipulation of ionic strength in solution. Each table has a corresponding figure.

Table 16

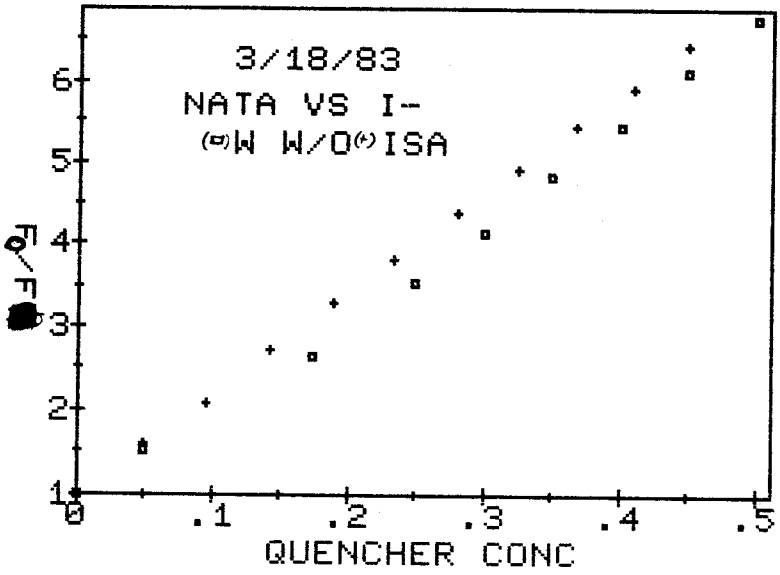
NATA vs. I^-

WITH IONIC STRENGTH ADJUSTOR (0.5M NaCl)			WITHOUT IONIC STRENGTH ADJUSTOR		
$[I^-]$	Avg F_o/F *		$[I^-]$	Avg F_o/F *	
0	1	---	0	1	---
.050	1.49	.01	.049	1.57	.01
.175	2.65	.02	.097	2.07	.08
.250	3.53	.02	.144	2.71	.03
.300	4.12	.02	.190	3.27	.02
.350	4.81	.06	.234	3.82	.04
.400	5.43	.08	.280	4.37	.02
.450	6.10	.10	.324	4.91	.02
.500	6.76	.02	.366	5.43	.05
---	---	-	.409	5.91	.06
---	---	-	.450	6.44	.01

* Avg of three runs.

The figure on the following page is a SV plot of both sets of data contained in the table above.

Figure 6



(ISA = NaCl)

Table 17

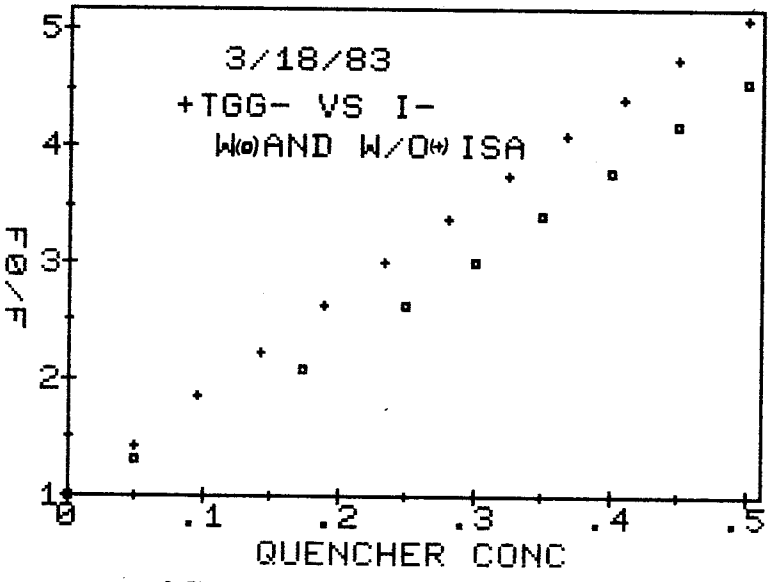
$^{+}TGG^{-}$ vs. I^{-}

WITH IONIC STRENGTH ADJUSTOR (0.5M NaCl)			WITHOUT IONIC STRENGTH ADJUSTOR		
$[I^{-}]$	Avg F_o/F *		$[I^{-}]$	Avg F_o/F *	
0	1	---	0	1	---
.050	1.31	.01	.049	1.42	.02
.175	2.09	.02	.097	1.86	.01
.250	2.63	.06	.144	2.22	.05
.300	2.99	.06	.190	2.62	.01
.350	3.41	.05	.234	3.01	.01
.400	3.79	.02	.280	3.36	.01
.450	4.19	.09	.324	3.74	.02
.500	4.54	.07	.366	4.10	.01
---	---	-	.409	4.42	.01
---	---	-	.450	4.74	.02
---	---	-	.491	5.09	.03

* Avg of three runs

This data is plotted in the SV plot on the following page. A substantial difference is seen between the two graphs.

Figure 7



0.5M
(ISA = NaCl)

Table 18

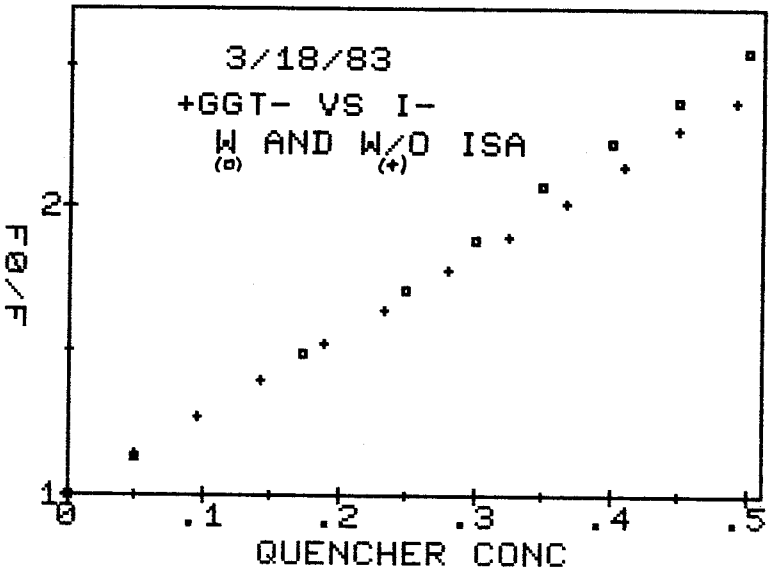
$^{+}GGT^{-}$ vs. I^{-}

WITH IONIC STRENGTH ADJUSTOR (0.5M NaCl)			WITHOUT IONIC STRENGTH ADJUSTOR		
$[I^{-}]$	Avg F_o/F *		$[I^{-}]$	Avg F_o/F *	
0	1	---	0	1	---
.050	1.12	.01	.049	1.13	.01
.175	1.48	.01	.097	1.26	.01
.250	1.71	.01	.144	1.39	.01
.300	1.88	.01	.190	1.52	.01
.350	2.06	.01	.234	1.64	.01
.400	2.22	.01	.280	1.77	.01
.450	2.37	.03	.324	1.89	.01
.500	2.54	.01	.366	2.01	.01
---	---	-	.409	2.14	.01
---	---	-	.450	2.26	.01
---	---	-	.491	2.37	.01

* Avg of three runs.

The SV plot which results from this data can be viewed on the next page. There is not a great difference between the two plots.

Figure 8



(ISA = $^{0.5M}_{NaCl}$)

Table 19

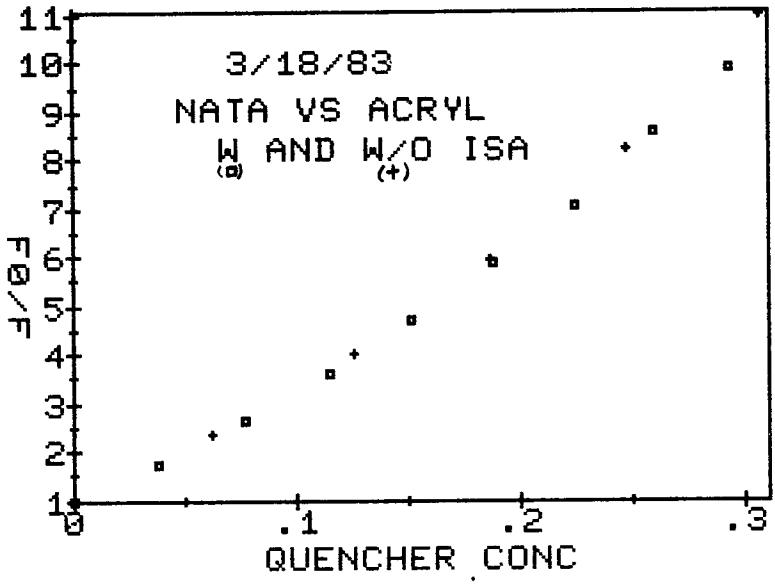
NATA vs. Acrylamide

WITHOUT IONIC STRENGTH ADJUSTOR			WITH IONIC STRENGTH ADJUSTOR (0.5M NaCl)		
[Acryl]	Avg F_o/F *		[Acryl]	Avg F_o/F **	
0	1	---	0	1	----
.063	2.32	.01	.039	1.75	.02
.126	4.01	.05	.077	2.61	.01
.187	5.98	.05	.115	3.61	.05
.247	8.26	.02	.152	4.71	.02
.306	11.00	.01	.188	5.91	.01
---	---	-	.224	7.04	.05
---	---	-	.259	8.57	.22
---	---	-	.293	9.92	.13

* Avg of five runs.
** Avg of three runs.

The accompanying SV plot can be found on the next page. There seems to be no noticeable difference between the two plots. Because both fluorophore and quencher are neutral molecules, an ionic strength effect would not be expected.

Figure 9



(ISA = 0.5M NaCl)

Table 20

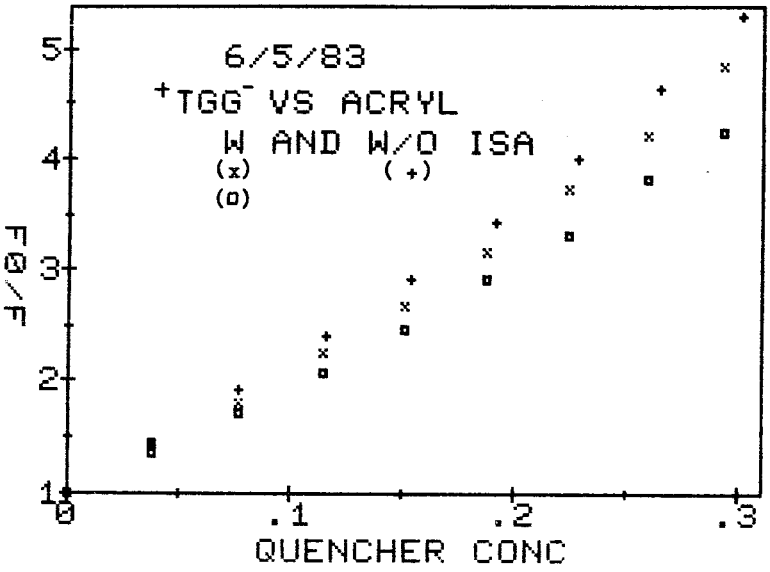
$^{+}TGG^{-}$ vs. Acrylamide

WITHOUT IONIC STRENGTH ADJUSTOR			WITH IONIC STRENGTH ADJUSTOR (0.5M NaCl)			WITH IONIC STRENGTH ADJUSTOR (0.5M KCl)		
[Acryl]	Avg F_o/F *		[Acryl]	Avg F_o/F *		[Acryl]	Avg F_o/F **	
0	1	---	0	1	---	0	1	---
.039	1.45	.01	.039	1.35	.01	.039	1.43	.01
.078	1.92	.02	.077	1.71	.01	.077	1.80	.03
.116	2.41	.03	.115	2.08	.01	.115	2.26	.01
.154	2.91	.01	.152	2.47	.04	.152	2.67	.01
.192	3.43	.06	.188	2.90	.02	.188	3.16	.05
.229	4.00	.08	.224	3.31	.02	.224	3.74	.01
.265	4.63	.08	.259	3.83	.01	.259	4.22	.01
.301	5.29	.08	.293	4.23	.01	.293	4.83	.01

*Avg of three runs
** Avg of two runs

The results listed in the table above are contained in the Stern-Volmer plot on the following page. This particular peptide was tested with the ionic strength adjustors NaCl and KCl.

Figure 10



x = 0.5M KCl

□ = 0.5M NaCl

Table 21

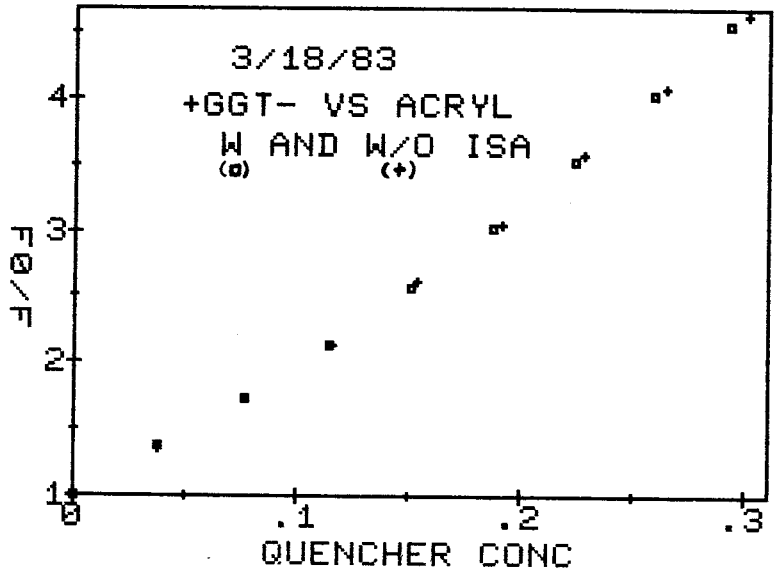
$^{+}GGT^{-}$ vs. Acrylamide

WITHOUT IONIC STRENGTH ADJUSTOR			WITH IONIC STRENGTH ADJUSTOR (0.5M NaCl)		
[Acryl]	Avg F_o/F *		[Acryl]	Avg F_o/F *	
0	1	---	0	1	---
.039	1.34	.01	.039	1.36	.01
.078	1.71	.01	.077	1.73	.01
.116	2.14	.03	.115	2.14	.01
.154	2.60	.03	.152	2.57	.02
.192	3.03	.04	.188	3.01	.03
.229	3.57	.05	.224	3.51	.03
.265	4.08	.06	.259	4.02	.06
.301	4.62	.05	.293	4.56	.03

* Avg of three runs

This data is plotted in a Stern-Volmer plot on the following page. There is not very much difference between the two sets of data.

Figure 11



0.5M
(ISA = NaCl)

Table 22

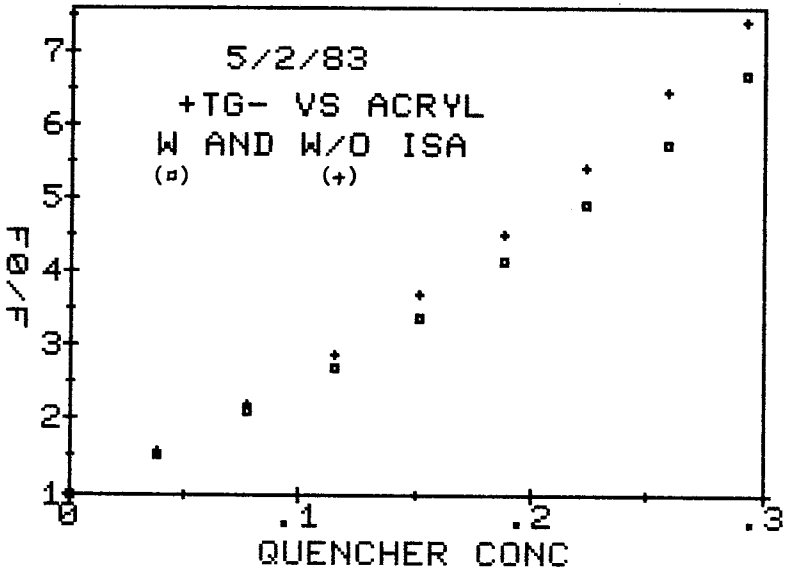
$^{+}TG^{-}$ vs. Acrylamide

WITHOUT IONIC STRENGTH ADJUSTOR			WITH IONIC STRENGTH ADJUSTOR (0.5M KCl)		
[Acryl]	Avg F_0/F **		[Acryl]	Avg F_0/F *	
0	1	---	0	1	---
.039	1.57	.01	.039	1.52	.01
.078	2.19	.02	.077	2.09	.01
.116	2.88	.05	.115	2.70	.01
.154	3.67	.07	.152	3.38	.01
.192	4.60	.08	.188	4.16	.05
.229	5.50	.08	.224	4.89	.06
.265	6.52	.09	.259	5.73	.06
.301	7.53	.13	.293	6.66	.08

* Avg of three runs
** Avg of five runs

The Stern-Volmer plot containing this data is found on the following page. A rather significant difference is noted between the two curves.

Figure 12



(ISA = 0.5M KCl)

Table 23

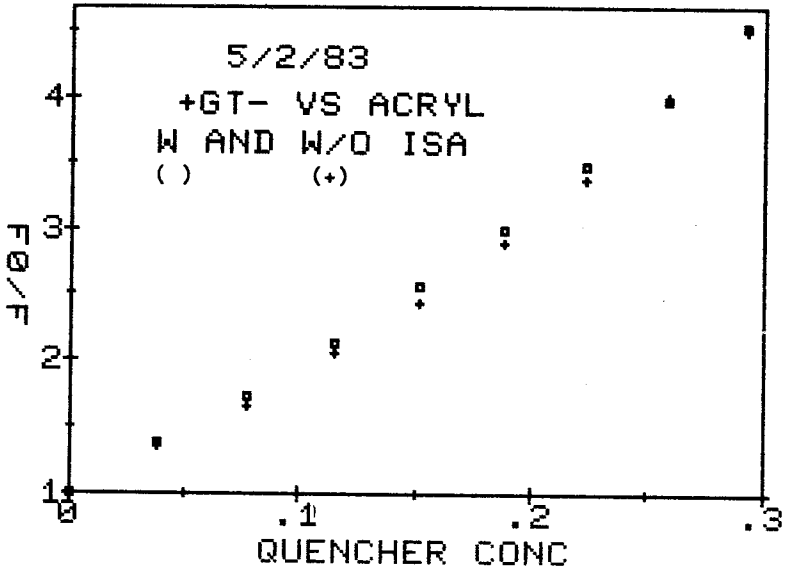
$^{+}$BT^{-} vs. Acrylamide

WITHOUT IONIC STRENGTH ADJUSTOR			WITH IONIC STRENGTH ADJUSTOR (0.5M KCl)		
[Acryl]	Avg F_0/F *		[Acryl]	Avg F_0/F *	
0	1	---	0	1	---
.039	1.33	.01	.039	1.36	.01
.078	1.65	.01	.077	1.73	.01
.116	2.06	.01	.115	2.23	.01
.154	2.44	.01	.152	2.55	.04
.192	2.90	.01	.188	3.00	.07
.229	3.36	.01	.224	3.48	.04
.265	4.00	.02	.259	3.99	.07
.301	4.51	.05	.293	4.54	.06

*Avg three runs

The Stern-Volmer plot for this data can be found on the next page. The two curves do not differ significantly from each other.

Figure 13



(ISA = 0.5M KCl)

Discussion

A. Relative Quantum Yield ϕ_R Determinations.

Relative quantum yield determinations were performed on all peptides used in fluorescince quenching experiments. (Table 8). N-acetyl-L-tryptophanide (NATA) was used as the reference fluorophore. The ϕ_R measurements served two purposes: they indicated which peptides' exhibted the greatest degree of fluorescence, and they gauged the "freshness" of peptides which had been stored in deep freeze for a lengthy period of time. Values of ϕ_R obtained were compared to previously determined values found in the literature.[REF 2]

B. Fluorescence Quenching Data: Peptides vs. Iodide and Peptides vs. Acrylamide

For fluorescence quenching studies on peptides versus iodide and versus acrylamide, two quencher concentration ranges were examined. In the earlier stages of experimentation, quencher concentration ranges of 0-0.25$\underline{M}$ I^- for iodide quenching and 0-0.212$\underline{M}$ acrylamide quenching were implemented. For iodide quenching experiments, ionic strength was maintained at 0.25 by a KCl ionic strength adjuster.

In later experiments, it was decided to extend both quencher concentration ranges. At higher quencher concentrations more deviation from classical Stern-Volmer behavior would be seen, and this data could be used to test the credibility of other quenching theories which tried to explain this deviation. The extended quencher concentration range for iodide was 0-0.50 $\underline{M}$, while that for acrylamide was 0-0.293$\underline{M}$. For ionic strength

control (0.500) in iodide quenching studies, a 5.0M NaCl solution was used.

All experimental results were presented in Stern-Volmer plots. These plots were made by plotting F_0/F versus $[Q]$, where F_0 represents fluorescence of a peptide solution in the absence of quencher, F represents fluorescence of a peptide solution in the presence of quencher, and $[Q]$ represents quencher concentration. The classical Stern-Volmer equation predicted that a straight line would result from a plot of F_0/F versus $[Q]$; however, definite upward curvature was consistently observed in all Stern-Volmer plots. It became obvious that the quenching mechanism could not be explained adequately by the classical Stern-Volmer equation. Therefore, in light of experimental results, other theories on the fluorescence quenching mechanism were sought. One theory selected for study was the modified Stern-Volmer static quenching model.

C. Fluorescence Quenching Data Analysis via the Modified Stern-Volmer Static Quenching Model.

At the heart of this theory is the presence of a static quenching volume around the fluorophore. (Figure 2). If a quencher molecule has interaction with this static quenching volume when a fluorophore becomes electronically "excited", it causes an immediate quench of the fluorophore. Therefore, it is necessary to know the probability that a quencher molecule will lie in the static quenching volume at a given quencher concentration. With this in mind, the following equation was derived:

$$\frac{F_o}{F\, e^{V[Q]}} = 1 + K_{sv}[Q]. \qquad (22)$$

In this equation, the variable V represents the static quenching volume of the fluorophore. Values of V and K_{sv} were obtained using a non-linear least square fitting program, and fluoroescence quenching data (F_o/F and [Q] values) were inputs for the program. After the input of a range of V values, which were chosen as probable volume values, the program would draw the best straight line through the data points for each given V value, which gave the the best straightline fit of the experimental data. A K_{sv} value was also obtained from this program. The computed values of K_{sv} and its corresponding V value for each peptide can be found in Table 14.

Comparing the Stern-Volmer plots of experimental data with the results presented in this table reveals that the Stern-Volmer plots showing the greatest amount of curvature also yield the highest V values. In order to explain curvature of this magnitude, a V value must be selected which is much greater than the contact volume of the fluorophore and quencher. Thus it would appear that physical contact between fluorophore and quencher is not necessary for a quench to occur.[REF 7] Because the static quenching model seemed physically unrealistic, fluorescence quenching data was used to investigate the Peak dynamic quenching model.

D. Fluorescence Quenching Data Analysis via the Peak Dynamic Quenching Model.

The Peak model focuses on the "pair" concentration of

fluorophore and quencher (see Figure 3) present in solution. In this model, an excited fluorophore may be quenched by its pair conjugate or by a nearby quencher which diffuses into the fluorophore.[REF 3] However, there must be physical contact between fluorophore and quencher for a quench to occur.

The actual mathematical derivation of the Peak equation for dynamic quenching is beyond the scope of this paper. Nevertheless, the results obtained by fitting experimental quenching data with the Peak model are significant. The fitting program yields a single parameter, ϵ, the quenching efficiency, which is an indicator of how well a fluorophore is quenched by a quencher molecule. The value for ϵ can range from 0 to 1, where a value of 1 means that a quench occurs for each fluorophore-quencher interaction. Through analysis of ϵ values for several peptides, it is possible to observe some relationship between peptide structure and ease of quenching.

Table 15 contains the results obtained when several peptides were fit to the Peak model. Much information can be gathered from this list. The first peptide listed is NATA. Analysis of all NATA data reveals that under similar conditions, acrylamide seems to be a more efficient quencher than iodide. Quenching efficiency data also seems to indicate charge effects. Observation of iodide quenching data shows that quenching efficiency of peptides by iodide increases as follows: $^{+}GGT^{-}$, $^{+}GT^{-}$, NATA, $^{+}GTG^{-}$, $^{+}GTGG^{-}$, $^{+}TG^{-}$, and $^{+}TGG^{-}$. This order correlates well with electrostatic arguments. The lowest quenching efficiencies occur when the negative charge is on trypt-

ophan and the highest efficiencies occur when the positive charge is on tryptophan. Intermediate quenching efficiencies result when the tryptophan carries no charge.

The Peak model explained the upward curvature found in a classical Stern-Volmer plot.[REF 3] It also detected a charge effect on quenching in the case of iodide quenching. The latter half of the research project was devoted to the study of charge effects and the effect of high ionic strength in solution on fluorescence quenching.

F. Fluorescence Quenching Data from Ionic Strength Manipulation Experiments.

In all Stern-Volmer plots presented, there is some degree of upward curvature. This indicated that the simple Stern-Volmer equation fails to incorporate all the parameters which influence the quenching mechanism. More "physical" aspects of the quenching mechanism must be investigated. Physical aspects include solvent-solute interactions and solute-solvent interactions, as occur in a solution whose ionic strength is controlled. Therefore, the quenching of several peptides was examined with and without ionic strength control. Each peptide and its Stern-Volmer plot will be discussed separately.

Figure 4 contains data on NATA versus I^- ionic strength experiments. Covering approximately the same quencher concentration range, two types of quenching tests were executed. In one test, the solution's ionic strength was maintained at 0.500M with NaCl, while in the other test, no ionic strength control was exercised on the solution.

NATA is a neutral molecule and will not electrostatically attract any charged species. Iodide, however, possesses a negative charge and will have an affinity for positively charged species. In a solution containing only NATA, iodide, and potassium, the efficiency of quenching is greater than in a solution containing NATA, iodide, potassium, sodium, and chloride. (NaCl = Ionic Strength Adjustor).

In order for quenching to occur, the iodide ion must collide with the NATA molecule. In solution without ionic strength control, the iodide ion is not very hindered in its approach to the NATA molecule. However, in a solution with ionic strength control, the microenvironment of the fluorophore-quencher pair is different. Although NATA still feels no attraction to charged species, the iodide quencher is now surrounded by sodium cations. Also, electrostatic interactions in solution are markedly increased. As a result of this increased effect on the iodide quencher, the efficiency of quenching should decrease. This is exactly what is seen in Figure 6. The quenching in solution containing ionic strength adjustor is less efficient than that in solution not containing ionic strength adjustor.

The results of quenching studies on $^{+}TGG^{-}$ versus I^{-} are plotted in Figure 7. In this case, tryptophan carries a positive charge. Thus, it shall attract negatively charged species. In a solution without ionic strength control, the attraction between fluorophore and quencher is quite strong, since the iodide possesses a negative charge. Therefore, efficiency

of quenching is quite good. However, in a solution containing ionic strength adjustor, the large number of ions in solution will inhibit the attraction and contact of the iodide and tryptophan. This ionic interference will manifest itself in a decrease in quenching efficiency. Figure 7 supports this view.

For $^{+}GGT^{-}$ versus I^{-}, the tryptophan residue possesses a negative charge and will attract species with positive charge. In a solution without ionic strength adjustor, fluorophore-quencher collisions do not encounter significant interference; however, the degree of quenching is not very large. In solution with ionic strength adjustor, there is not much deviation from the results obtained in solution without ionic strength adjustor until greater quencher concentrations are encountered. Greater quenching efficiency at greater quencher concentrations could be attributed to the excess ions in solution which help to nullify the electrostatic repulsion of the negatively charged fluorophore and quencher. As a result, quenching efficiency increases, as is seen in Figure 8.

Several peptides were also quenched by acrylamide, a neutral molecule. Because both NATA and acrylamide are neutral moieties, manipulation of the ionic strength of the test solution had no substantial effect on quenching efficiency. This is seen in Figure 9.

Unexpectedly, an ionic strength effect is found in $^{+}TGG^{-}$ versus acrylamide experiments. The efficiency of quenching in a solution with ionic strength adjustor is markedly less

than the efficiency of quenching in solutions without ionic strength adjustor. Even more startling is the fact that the efficiency of quenching appears to be dependent on which ionic strength adjusting salt is used. In one set of experiments NaCl is used, while in another, KCl is used. The NaCl decreases quenching efficiency more than KCl does. A "cation" effect seems to be present. In either case, the added ions in solution inhibit the rate of collision of fluorophore and quencher, and therefore, the efficiency of quenching is less in a solution with ionic strength adjustor. Results of these experiments are found in Figure 10.

For tests on $^{+}GGT^{-}$ versus acrylamide, no significant ionic strength effect is observed. (See Figure 11).

Two other peptides were also tested versus acrylamide: $^{+}TG^{-}$ and $^{+}GT^{-}$. The ionic strength adjusting salt used was KCl. The effects of ionic strength manipulation on the quenching of these peptides by acrylamide parallel the ionic strength effects observed on the quenching of $^{+}TGG^{-}$ and $^{+}GGT^{-}$. For $^{+}TG^{-}$ versus acrylamide, the efficiency of quenching is greater for a solution without ionic strength adjustor (KCl) than for a solution without ionic strength adjustor. (Figure 12). As for $^{+}GT^{-}$ versus acrylamide, there is no significant difference in quenching efficiency of solutions with or without ionic strength adjustor.

From these correlations, it is apparent that charges on fluorophore, quencher, or on solution ions do affect the quenching mechanism. Therefore, subsequent quenching experiments,

test solutions should contain a specific ionic strength adjustor to insure consistency and reproducibility of data.

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(5) Turro, Nicholas J., Molecular Photochemistry, W.A. Benjamin, Inc., New York, 1965.

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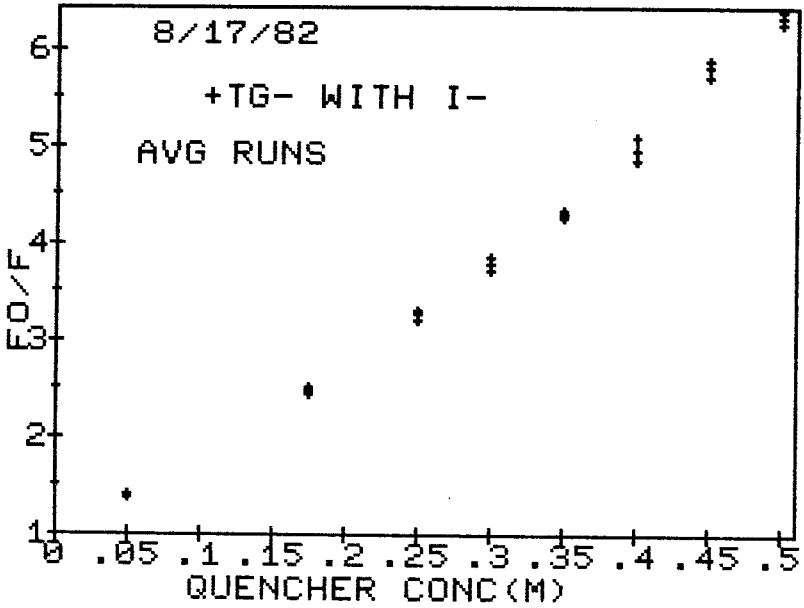
(7) Peak, David, preprint, "The Role of Competition in the Quenching of Fluorescence by Collisional Contact".

(8) Parker, C.A., Photoluminescence of Solutions, Elsevier, New York, 1968.

APPENDIX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+TG- WITH I-
AVG RUNS
Fo/F
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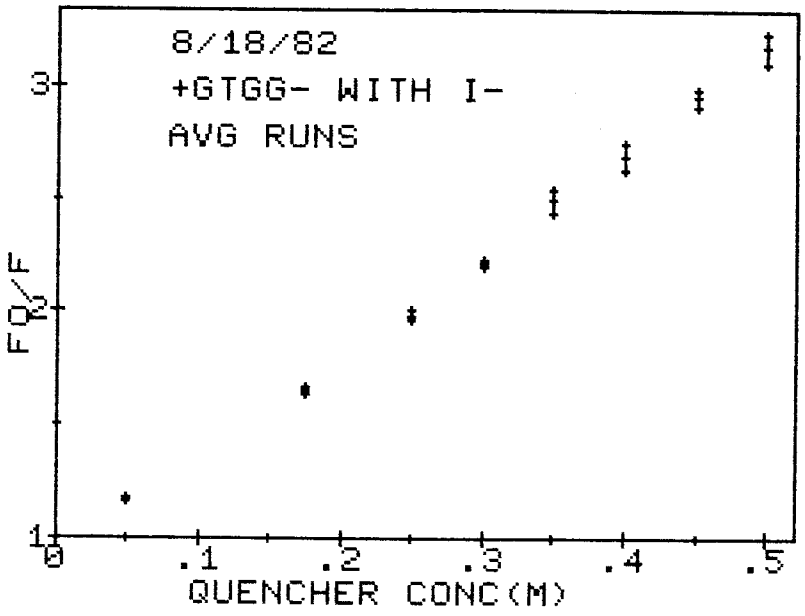
5
4
3
2
1
0 .05 .1 .15 .2 .25 .3 .35 .4 .45 .5
QUENCHER CONC(M)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.3
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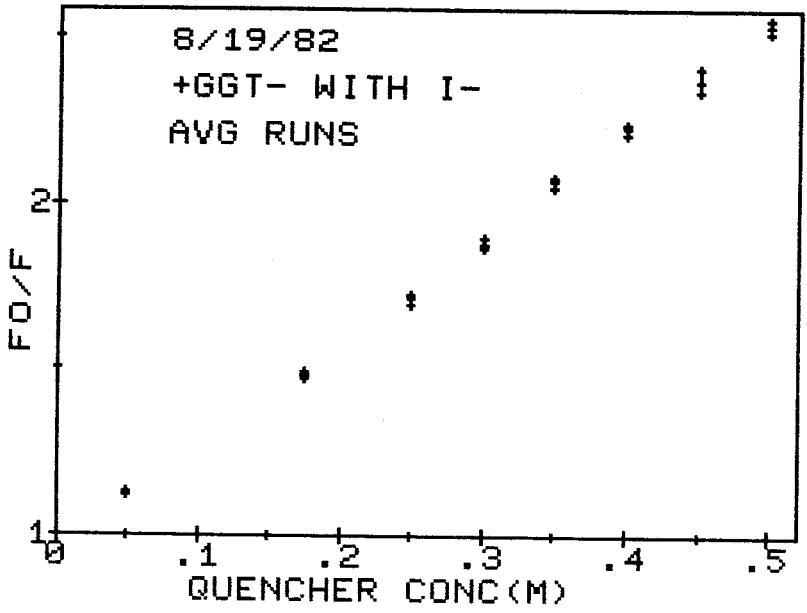
.5
QUENCHER CONC(M)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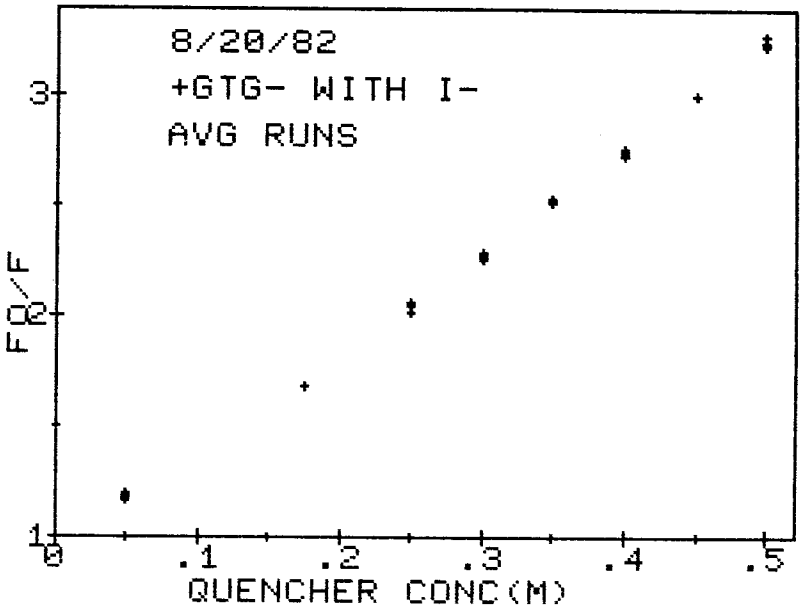
.2
.3
.4
.5
QUENCHER CONC(M)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FO/F
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.2
.3
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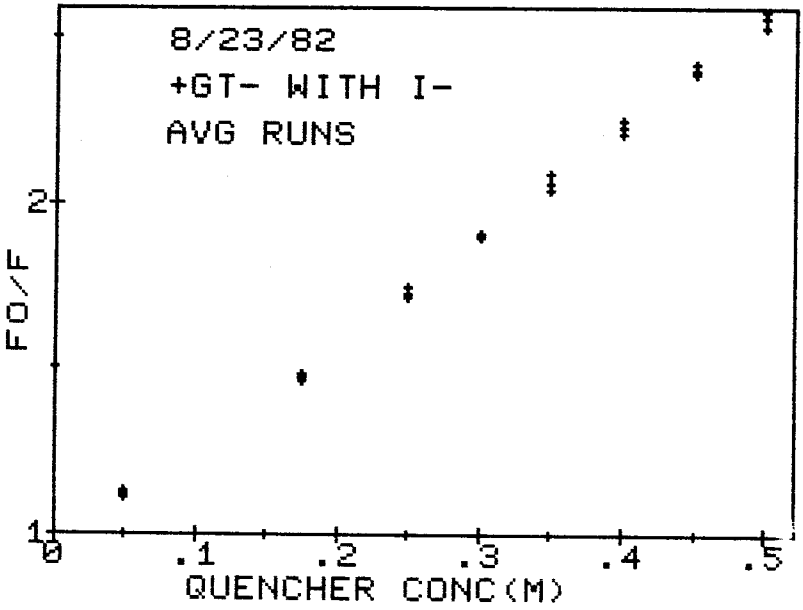
QUENCHER CONC(M)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FO/F
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.2
.3
.4
.5
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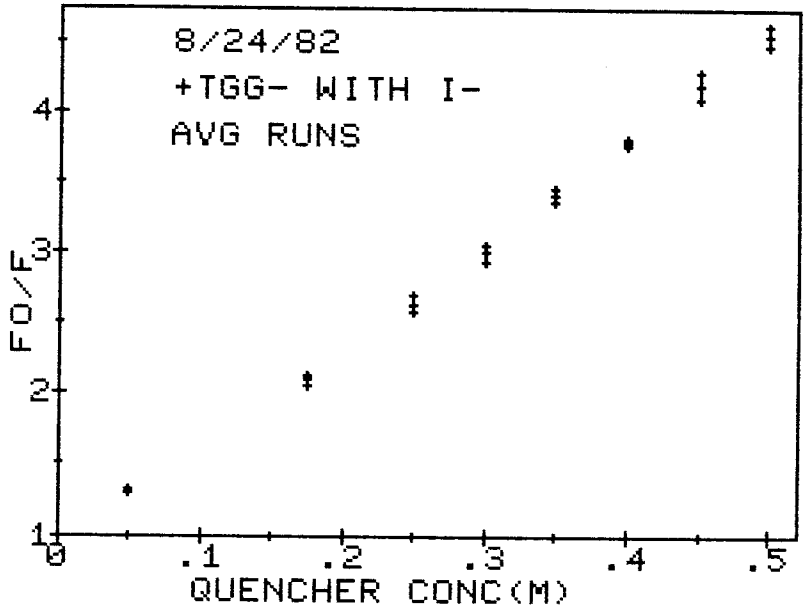
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.2
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.4
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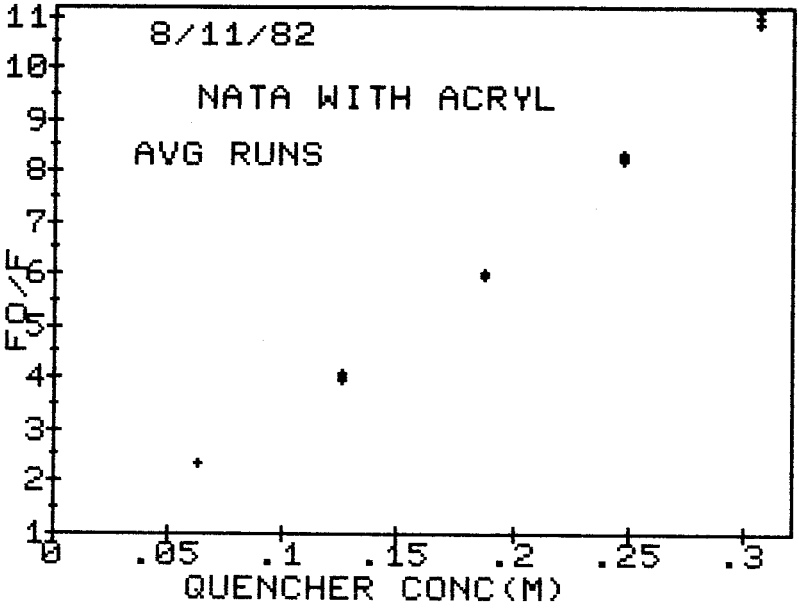
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NATA WITH ACRYL
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10
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8
7
6
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2
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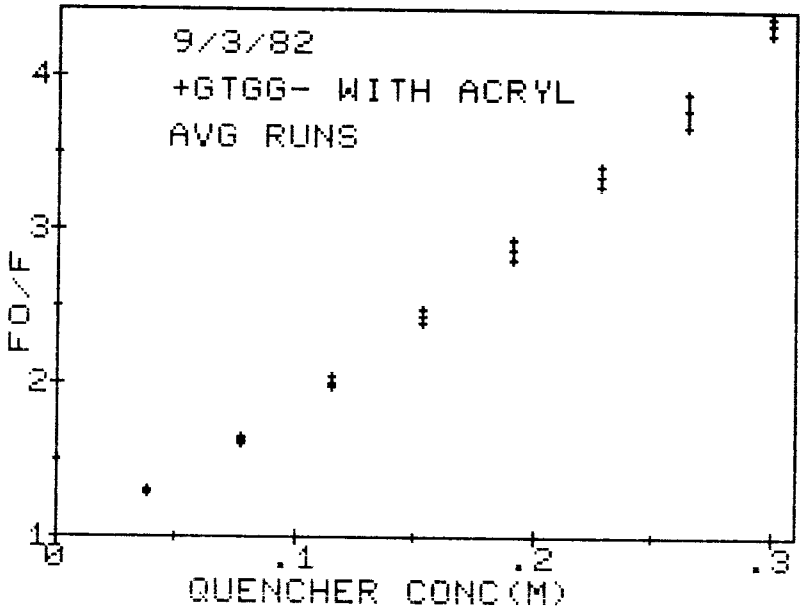
.1
.15
.2
.25
.3
QUENCHER CONC(M)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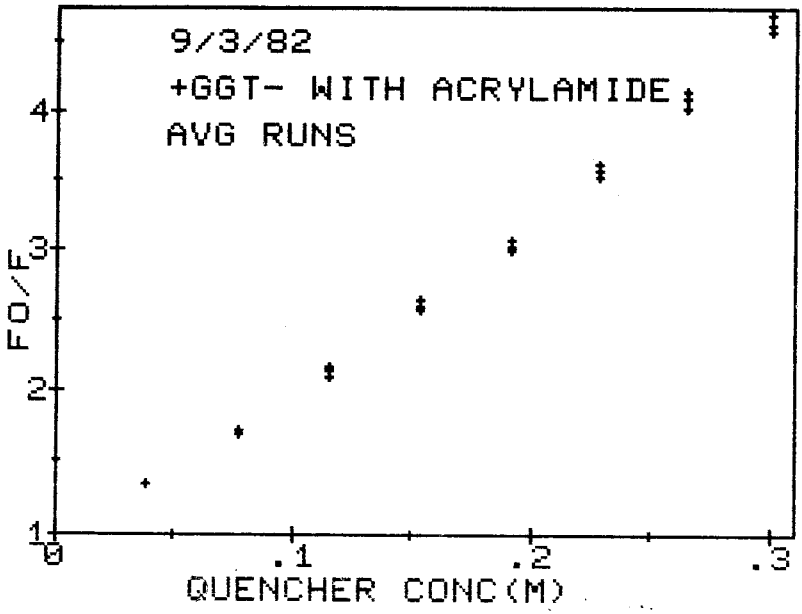
.1
.2
.3
QUENCHER CONC(M)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4
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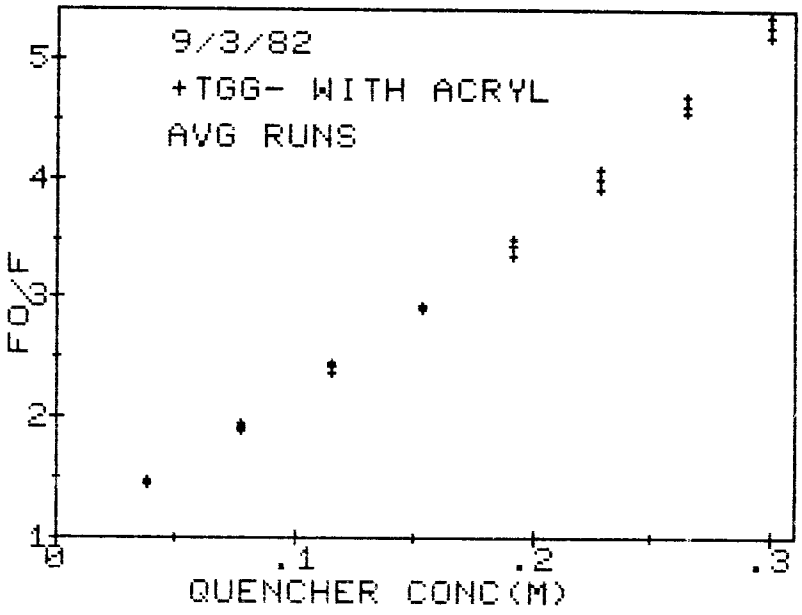
.1
.2
.3
QUENCHER CONC(M)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AVG RUNS
FO/F
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2
3
4
5
0
.1
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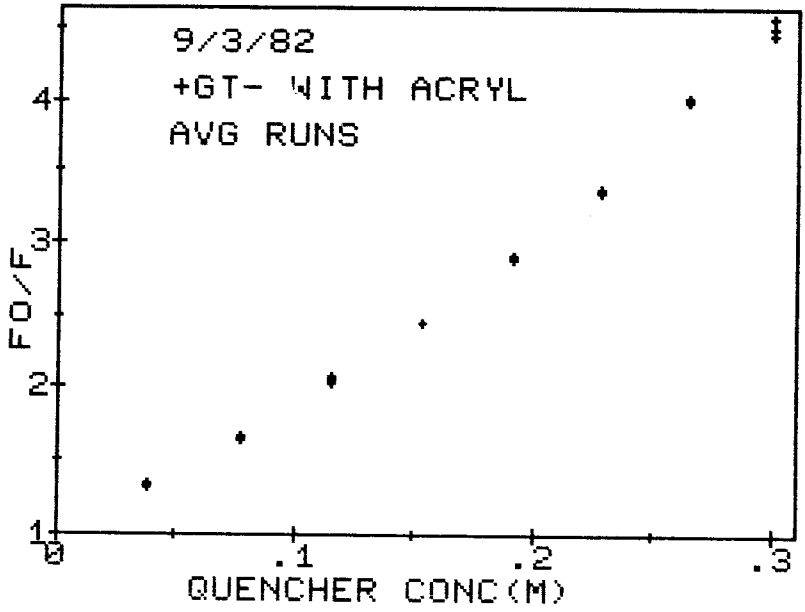
.3
QUENCHER CONC(M)


9/3/82
+GT- WITH ACRYL
AVG RUNS
FO/F
1
2
3
4
0
.1
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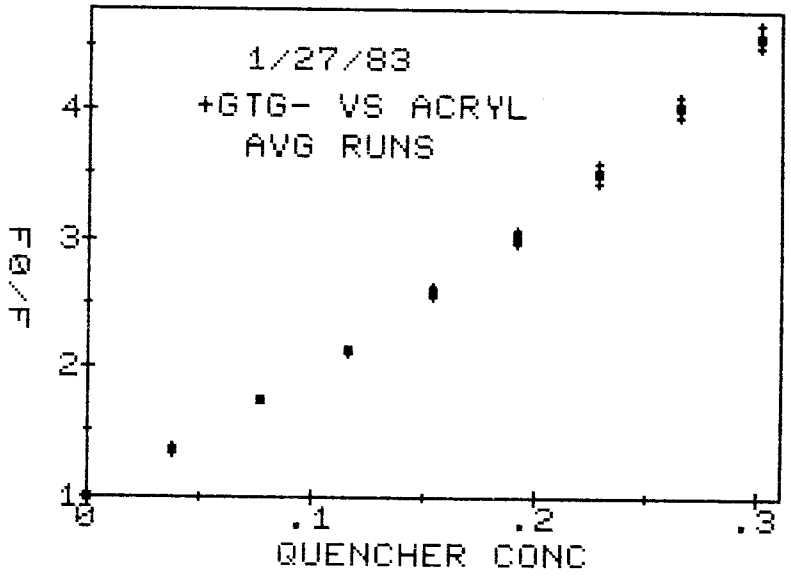
.3
QUENCHER CONC(M)


1/27/83
+GTG- VS ACRYL
AVG RUNS
F0/F
4
3
2
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0
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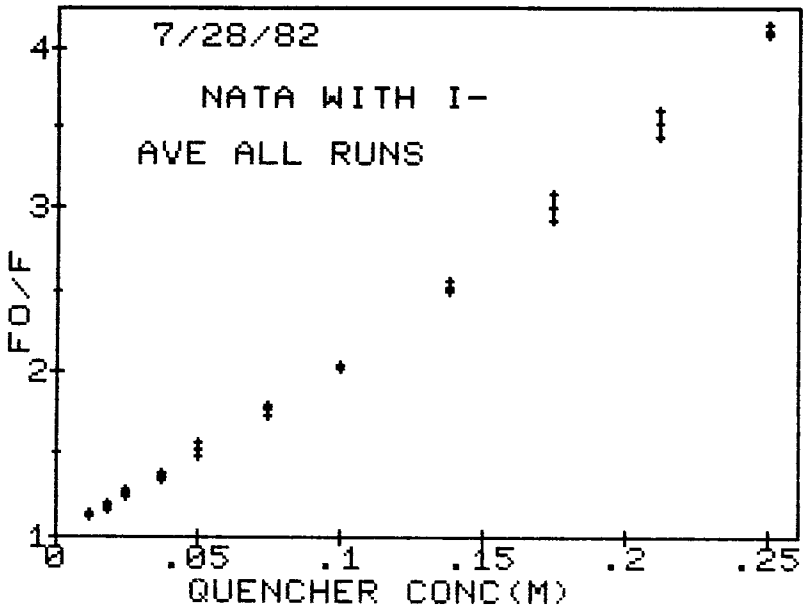
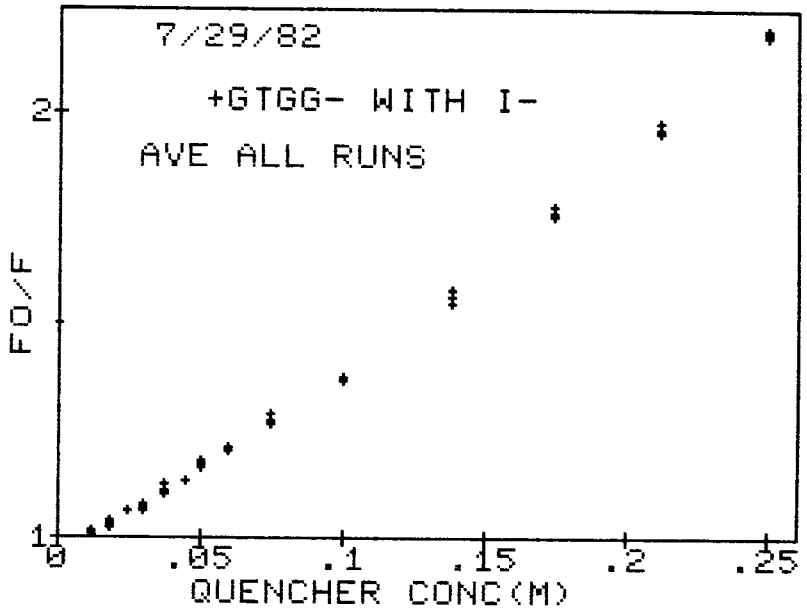
.3
QUENCHER CONC


7/28/82
NATA WITH I-
AVE ALL RUNS
FO/F
4
3
2
1
0
.05
.1
.15
.2
.25
QUENCHER CONC(M)


7/29/82
+GTGG- WITH I-
AVE ALL RUNS
FO/F
2
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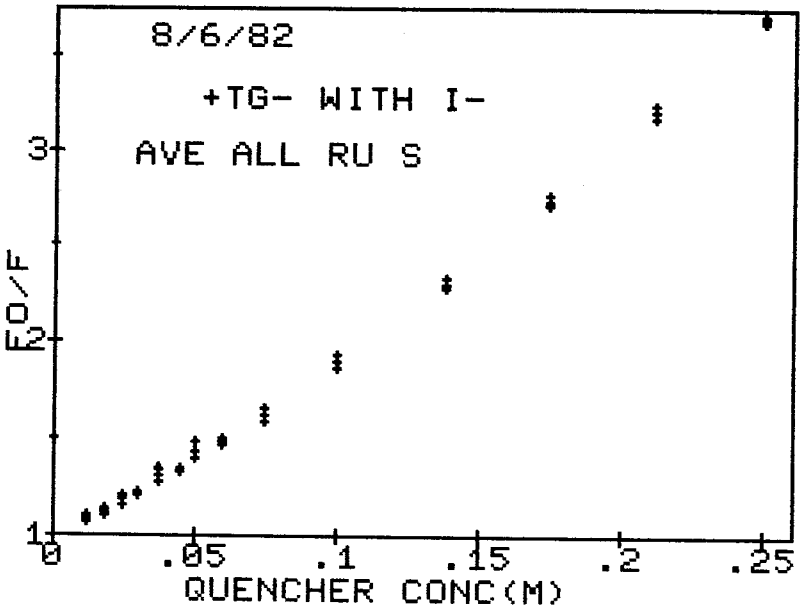
8/6/82
+TG- WITH I-
AVE ALL RU S
3
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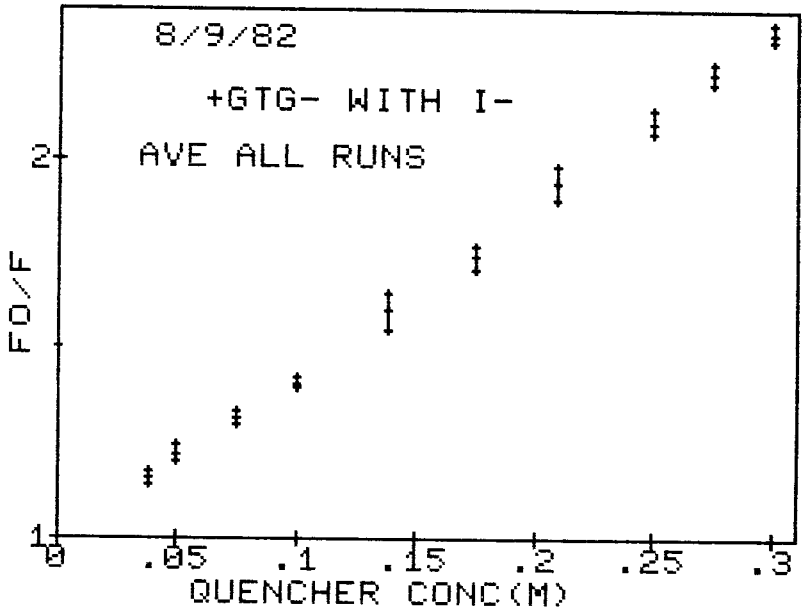
QUENCHER CONC(M)


8/9/82
+GTG- WITH I-
AVE ALL RUNS
FO/F
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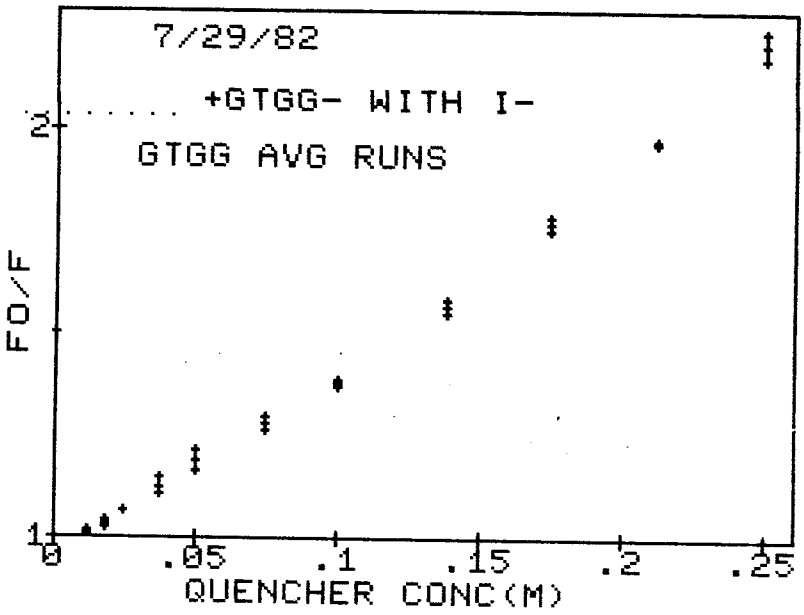
QUENCHER CONC(M)


7/29/82
+GTGG- WITH I-
GTGG AVG RUNS
FO/F
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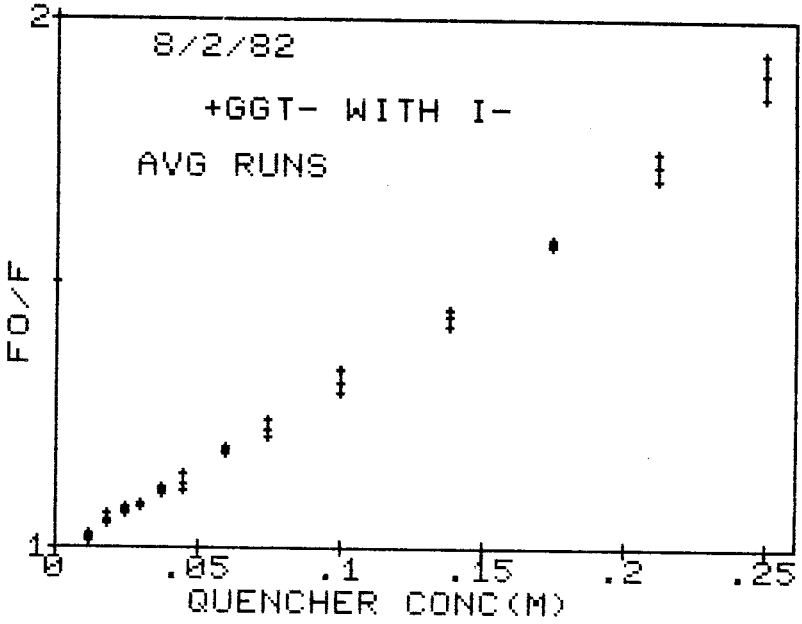
QUENCHER CONC(M)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AVG RUNS
FO/F
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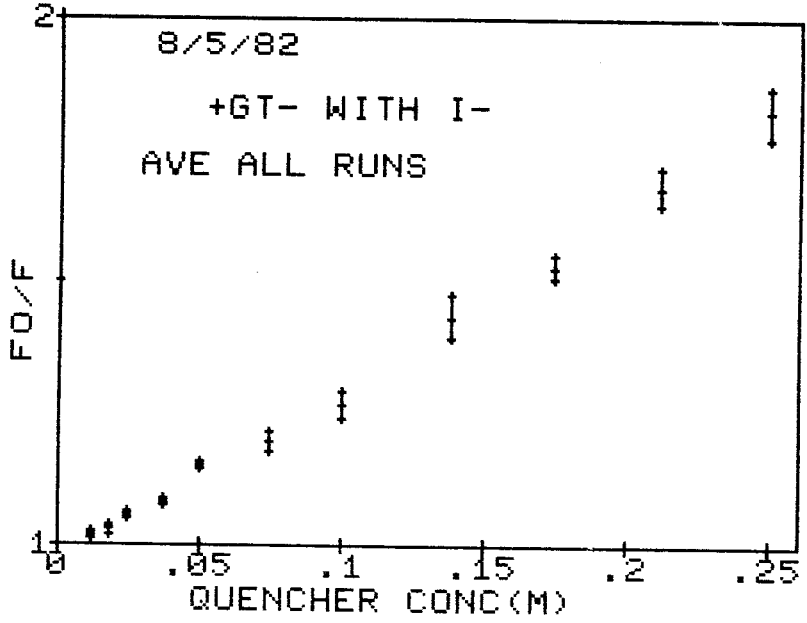
8/5/82
+GT- WITH I-
AVE ALL RUNS
FO/F
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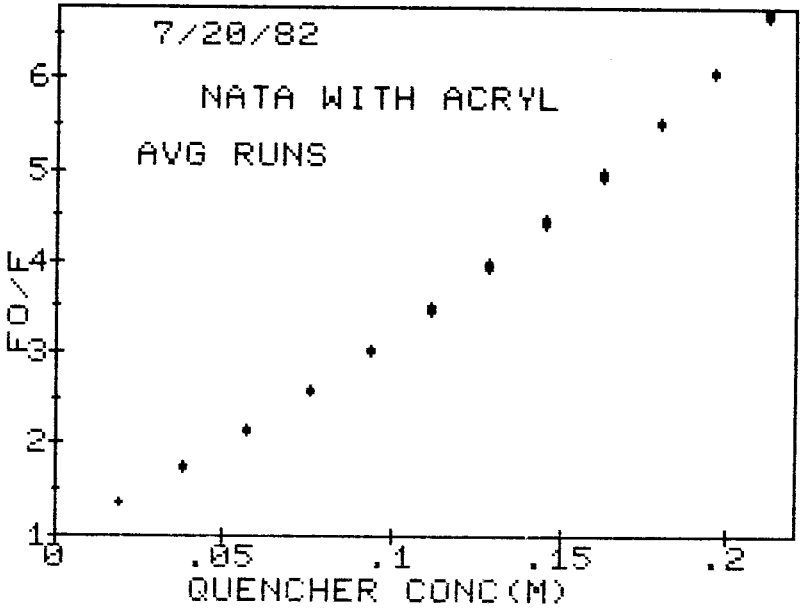
7/20/82
NATA WITH ACRYL
AVG RUNS
FO/F
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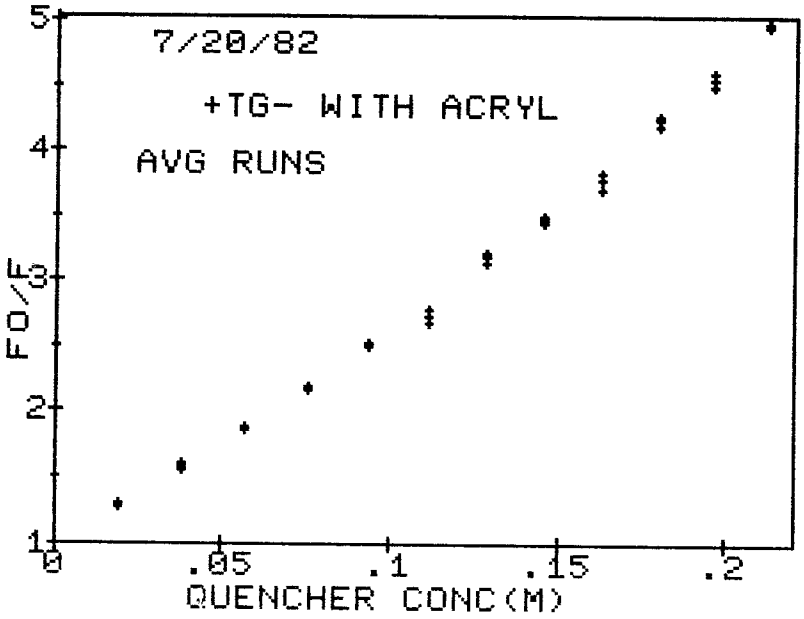
QUENCHER CONC(M)


7/20/82
+TG- WITH ACRYL
AVG RUNS
FO/F
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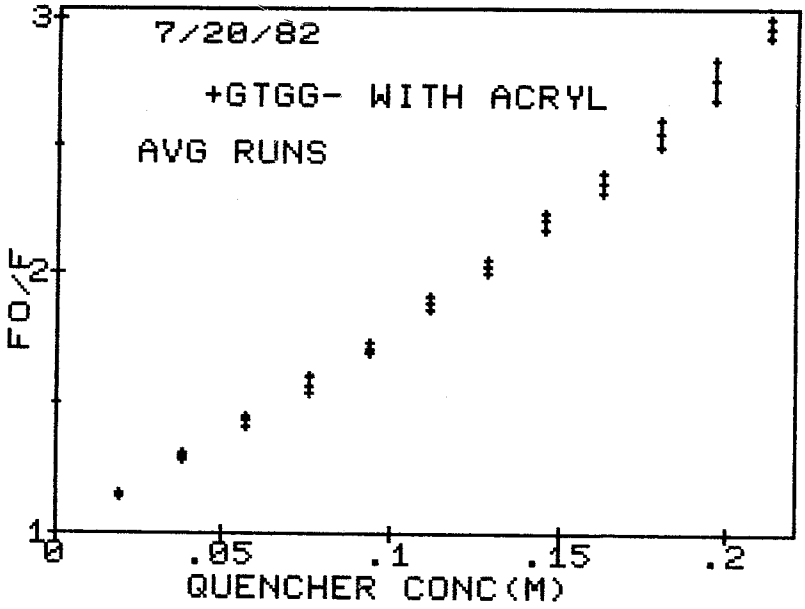
QUENCHER CONC(M)

UN82 D411f/1983 DENNIN, R. M. CHEMISTRY FLUORESCENCE QUENCHING OF , ETC. HRS. 6/83 SHT. 2 OF 2


7/20/82
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AVG RUNS
FO/F
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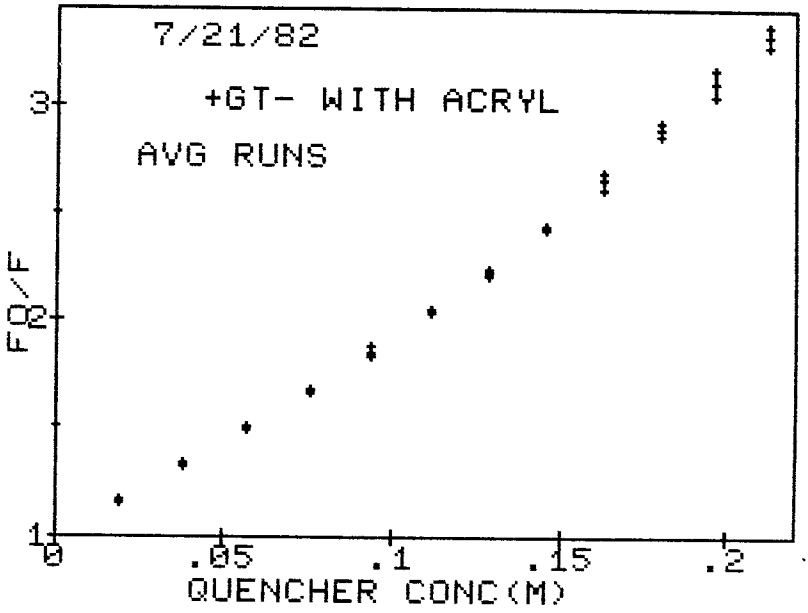
QUENCHER CONC(M)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FO/F
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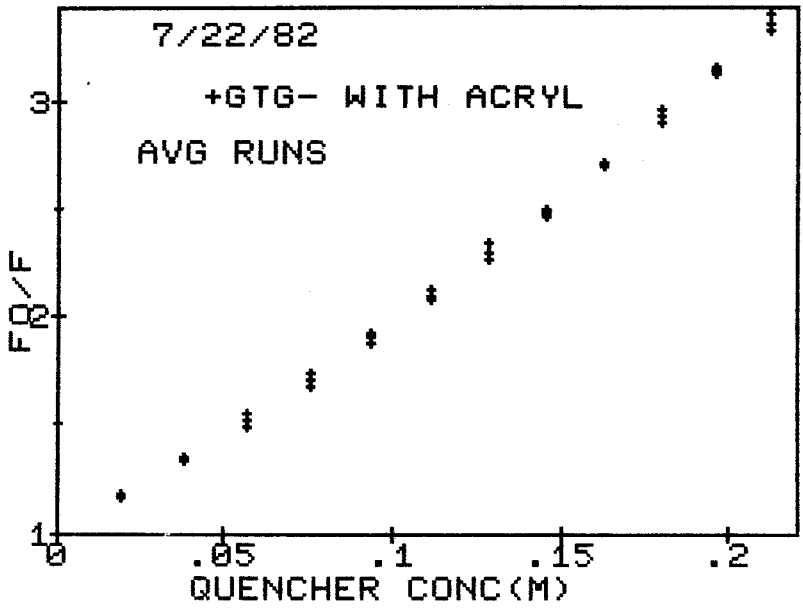
QUENCHER CONC(M)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AVG RUNS
FO/F
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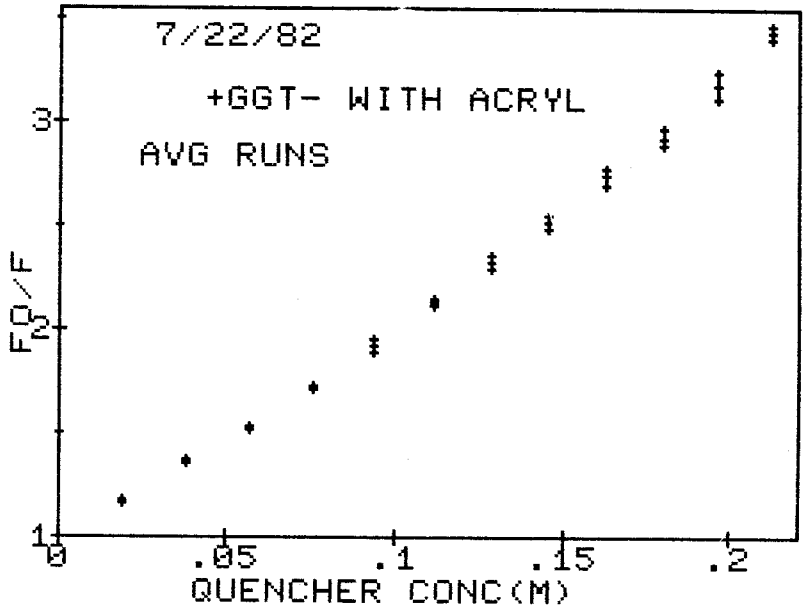
QUENCHER CONC(M)


7/22/82
+GGT- WITH ACRYL
AVG RUNS
FO/F
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QUENCHER CONC(M)

END