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The Magnitude of Charge Effects on the Fluorescence Quenching of Peptidyl Tryptophan by Iodide

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THE MAGNITUDE OF CHARGE EFFECTS ON THE
FLUORESCENCE QUENCHING OF PEPTIDYL
TRYPTOPHAN BY IODIDE

by

James L. Danziger

* * * * *

Submitted in partial fulfillment
of the requirements for
Honors in the Department of Chemistry

UNION COLLEGE

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1984ABSTRACT

DANZIGER, JAMES The Magnitude of Charge Effects on the Fluorescence Quenching of Peptidyl Tryptophan by Iodide. Department of Chemistry, May 1984.

The magnitude of charge effects on the quenching of tryptophan containing peptides by Iodide (I^-) is compared for those peptides containing a positively charged tryptophan to those peptides containing a negatively or uncharged tryptophan. The positively charged tryptophan residue can draw the negatively charged iodide quencher to it, and this charge effect increases the quenching of the tryptophan by as much as a factor of two as opposed to those cases where no charge effect occurs.

The fluorescence quenching of several tryptophan containing peptides by iodide is found to be dependent on the nature of the ionic strength adjuster (ISA) used to insure constant ionic strength in the quencher solution. This effect is seen if the tryptophan containing peptide is in the zwitterion form with a positive charge on the tryptophan and the ISA contains Cl^- . The positive charge draws the negatively charged chloride ion to it, allowing the Cl^- to exert a heavy atom type of quenching effect and thereby reducing the effective quenching by I^- . However, when the tryptophan has a negative charge on it, the Cl^- is not drawn to the tryptophan and, in this case,

no inhibition of the I- quenching is observed.

Therefore, sodium or potassium chloride, which in the past have commonly been used as ionic strength adjusters can inhibit I- quenching if there is a positive charge on the species which fluoresces. This invalidates the use of these salts as ionic strength adjusters.

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INTRODUCTION

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. These excited states can be singlet (S_1) 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 giving off a photon by either fluorescence (F) or phosphorescence (P) or by non-radiative processes such as internal conversion (IC) or intersystem crossing (ISC) (See Figure 1).

The electronic transitions for the population and depopulation of the singlet state can be expressed kinetically as follows:

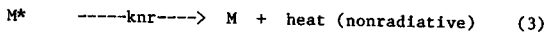
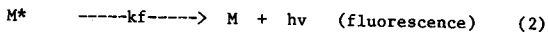
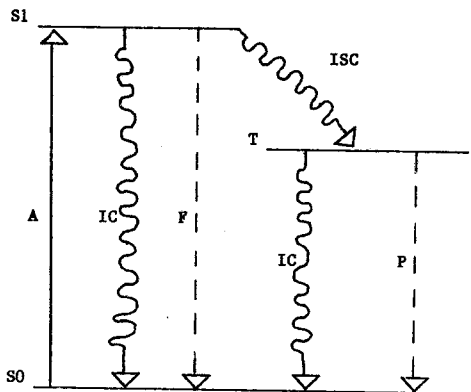


FIGURE 1JABLONSKI DIAGRAM (1)

S0 = ground electronic state

S1 = excited electronic state (singlet)

T = excited electronic state (triplet)

A = absorbance

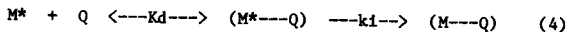
F = fluorescence

P = phosphorescence

IC = internal conversion

ISC= intersystem crossing

Another form of deactivation of the excited state, called fluorescence quenching, can occur upon introducing a quencher molecule into the system. It can be expressed as follows:⁽²⁾



where the encounter complex, $(M^* \cdots Q)$, that is formed with a diffusion controlled rate constant, k_d , reacts to dissipate the electronic energy by some internal mechanism with rate constant k_i . The rate constant for the entire process in (4) is k_q .

If a molecule is exposed to steady illumination, the rate of formation of the excited state molecules can be written as:⁽¹⁾

$$d\langle M^* \rangle / dt = k_e - (k_f + k_q \langle Q \rangle + k_{nr}) \langle M^* \rangle \quad (5)$$

Using the steady state assumption for $\langle M^* \rangle$ yields:

$$k_e = (k_f + k_q \langle Q \rangle + k_{nr}) \langle M^* \rangle \quad (6)$$

Given that the quantum yield (ϕ), or the photons emitted divided by the photons absorbed, for the emission from $\langle M^* \rangle$ in the absence of $\langle Q \rangle$ is:

$$\phi_0 = k_f \langle M^* \rangle / k_e = k_f / (k_f + k_{nr}) \quad (7)$$

and in the presence of $\langle Q \rangle$ is:

$$\phi_a = k_f \langle M^* \rangle / k_e = k_f / (k_f + k_q \langle Q \rangle + k_{nr}) \quad (8)$$

then the following ratio can be written:

$$\frac{\phi_0}{\phi_a} = \frac{k_f + k_q \langle Q \rangle + k_{nr}}{k_f + k_{nr}} = 1 + \frac{k_f}{k_f + k_{nr}} \langle Q \rangle \quad (9)$$

This result is the Stern-Volmer equation which describes collisional quenching under steady state conditions. This may also be written as:

$$\phi_0 / \phi_a = 1 + K_{sv} \langle Q \rangle = 1 + k_q T_o \langle Q \rangle = T_o / T_a \quad (10)$$

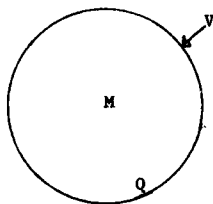
where ϕ_0 and ϕ_a are the yield in the absence and presence of quencher Q . K_{sv} is the Stern Volmer quenching constant and is equal to the rate constant for collisional quenching, k_q , times the lifetime, T_o , where T_o is the lifetime of fluorescence in the absence of quencher. By definition, the lifetime is the time it takes the population of excited states to decay to 37% of its initial value. A plot of ϕ_0 / ϕ_a versus $\langle Q \rangle$ yields a straight line with slope K_{sv} and intercept of 1. If the lifetime, T_o , is measured or known, the second order rate

constant k_q can be determined from the slope of the plot (since $K_{sv} = k_q \tau_0$). For this project plots of F_0/F versus $\langle Q \rangle$ were drawn where F_0 and F are the fluorescence intensities at a given wavelength in the absence and presence of quencher Q respectively. This is acceptable because the ratios of ϕ_0/ϕ_a and F_0/F are equal since the shape and wavelength maximum are unaffected by quencher.

Stern Volmer plots are often seen to curve upwards. These positive deviations can be explained by the fact that only a certain fraction of the excited states are actually quenched by a collisional mechanism. Some excited states are deactivated almost instantaneously after being formed because a quencher happens to be positioned in their proximity at the time they are excited.⁽²⁾ This type of quenching is called a "static" quench. It follows that as quencher concentration increases, the probability of static quenching increases. The modified Stern Volmer equation is derived using the static quenching model (See Figure 2).⁽²⁾ Any quencher which enters volume V will cause an immediate static quench of the excited fluorophore when its excited. By calculating the probability that a M will have a Q within V leads to the modified Stern Volmer equation:

$$F_0/F_e^{V[Q]} = 1 + K_{sv}\langle Q \rangle = \tau_0/\tau \quad (11)$$

where V is the static quenching constant.⁽²⁾ We empirically

FIGURE 2STATIC QUENCHING MODEL

Where M is the fluorophore, Q is the quencher, and V is the static quench volume.

Calculating the probability that a M will have a Q within V leads to the modified Stern Volmer equation:

$$F_0/F_e^{V[a]} = 1 + K_{sv}\langle Q \rangle$$

where V is the static quenching constant.

vary V until F_0/F_e^{val} versus $\langle Q \rangle$ gives a straight line. The slope of this line is equal to K_{sv} , and from that we can get k_q which is a measure of the dynamic quenching.

Fluorescence quenching studies are used for many purposes. One biochemical application is the use of fluorescence quenching to determine the accessibility of tryptophan residues in proteins by iodide (I^-) quenching of fluorescence. I^- is unable to penetrate into the interior of the protein. This means that iodide quenching could be used to determine the fraction of total fluorescence which is due to surface localized tryptophan residues.⁽³⁾ So that we can make accurate predictions of what is happening in these biochemical systems from quenching studies, it must be determined if any outside or secondary effects are causing inaccuracies in the data. The goal of this study is to determine the magnitude of charge effects in the quenching of charged tryptophan containing peptides.

Of the twenty amino acids present in proteins, only three exhibit fluorescence; tryptophan, tyrosine, and phenylalanine.⁽⁴⁾ Each contains an aromatic function which is responsible for the fluorescence. The the magnitude of tryptophan fluorescence is much greater than that of tyrosine or phenylalanine. The peptides selected for study contain a tryptophan residue and 0-2 glycine residues. These peptides are in the zwitterion form allowing for manipulation of positive and negative charge

on or around the tryptophan residues. These tryptophan-glycine peptides will be written in an abbreviated form, such as +TG-, +GT-, +TGG-, etc. The quencher molecules used bear a charge, and thus a charge effect can be studied.

We are looking for the magnitude of variation of quenching efficiency when we have a combination of a charged quencher molecule and charged tryptophan residue. We will evaluate these variations by using small peptides containing exposed tryptophan residues in water and hope to use this as a model for exposed tryptophans in larger proteins.

The type of quenching which is present in this study is heavy atom quenching.⁽³⁾ The quenching is short range requiring a momentary complex to be formed between the fluorophore and heavy atom quencher in which the orbital on the heavy atom can overlap those on the excited molecule.⁽⁵⁾ The quenching which is observed is a consequence of a heavy atom enhanced rate of intersystem crossing to the triplet state which competes with fluorescence decay.⁽⁵⁾

In quenching studies, the change in fluorescence intensity of a fluorophore with varying quencher concentration is observed. In this study, peptides containing charged tryptophan are being quenched with varying concentrations of iodide (I⁻). In order to insure that the peptides studied are in a "constant ionic

environment", ionic strength adjustors (ISA) are added.

One commonly used ISA is NaCl. A secondary goal of this study is to evaluate the role of commonly used ISA's, such as NaCl, in affecting quenching by I⁻.

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, either a Beckman DU or a Cary 118C spectrophotometer was used. In the Beckman DU the source is a hydrogen lamp while in the Cary 118C the source used is a deuterium lamp.

To obtain fluorescence emission data, a Hitachi Perkin Elmer Model MPF-2A spectrofluorometer was used. Excitation radiation from a xenon lamp at 295 nm was used and excitation and emission bandpasses were 6 nm and 7 nm, respectively.

In the preparation of the phosphate and bicarbonate buffer solutions an Orion Research Model 701/digital Ionalyzer was used to measure the pH of the solutions.

Finally, an Apple Computer was used for data analysis and was interfaced with the fluorometer. The interface of the computer with the fluorometer made it easier and quicker to take measurements. The computer

was also used to process experimental data and to print Stern Volmer plots of fluorescence data.

II. Solution Preparation

A. Buffer Solutions:

In order to keep test solutions at a pH of approximately 6.0, so that peptides would be in the zwitterion form, a .15 molar phosphate buffer was used. The buffer solution was prepared by adding 4.021 grams of Na_2HPO_4 to 100 ml's of distilled H_2O (dH_2O), and then adding HCl until a pH of 6.0 was achieved. A .15 molar $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$ buffer solution results.

In order to keep test solutions at a pH of 9.8, so that peptides would be in the anionic form, a .15 molar bicarbonate buffer was used. The buffer solution was prepared by adding 1.260 grams of NaHCO_3 to 100 ml's of dH_2O , and then adding an NaOH solution until a pH of 9.8 is achieved. A .15 molar $\text{HCO}_3^-/\text{CO}_3^{2-}$ buffer solution results.

B. Ionic Strength Adjusters:

Ionic strength adjusters are used in order to keep an observed peptide in a constant ionic environment. Control of the ionic strength was achieved by adding one of two ionic strength adjusters (ISA's). The two ISA solutions were prepared from either NaCl or NaF . For a

2.50 molar NaCl solution, 14.608 grams of NaCl were added to a 100 ml volumetric flask and diluted to the mark with dH₂O. A 0.0625 molar NaF solution was prepared in the same manner using 2.624 grams of NaF.

C. Iodide Quencher Solution:

The iodide quencher solution was prepared from NaI. A 5.0 molar NaI solution was prepared by adding 18.736 grams of NaI to a 25 ml volumetric flask and then filling to the mark with dH₂O. In order to stabilize the quencher and prevent the formation of I_3^- , a small amount (10^{-4} molar) of NaS₂O₃ was added.

D. Peptide Stock Solution:

For experimental runs, the peptide stock solution was prepared by adding enough solid peptide to approximately 12 ml's of dH₂O to achieve a solution with an absorbance between 0.8 and 1.2 at an excitation wavelength of 280 nm.

III. Procedure

A. Preparation of Solutions for Quenching Measurements:

The solutions used for these tests were a 0.15 molar phosphate buffer (pH 6.0), a 0.15 molar bicarbonate buffer (pH 9.8), peptide solution ($A_{280} = 0.8 - 1.2$), 2.5 molar NaCl (ISA), 0.625 molar NaF (ISA), and 5.0 molar

NaI (quencher).

For each iodide quencher concentration to be studied, a separate solution was prepared in a 10 ml volumetric flask. Iodide quencher concentration ranged from 0.0 to 5.0 molar. In order to achieve the same ionic strength in each solution, the proper amount of an ISA solution was added along with the proper amounts of buffer solution (protocol was the same for either the phosphate or bicarbonate buffer) and peptide solution to the 10 ml volumetric flasks followed by dilution to the mark with dH₂O (See Tables 1 and 2 for the exact protocol).

B. Determination of Fo/F Ratios:

Once the solutions were prepared, the actual fluorescence measurements could be made using the following procedure. The solution to be tested is pipetted into a fluorescence cell, the cell is inserted into the fluorometer, and the fluorometer settings are adjusted to the desired values. An Apple computer is interfaced with the fluorometer and a program which measures fluorescence intensity at a fixed wavelength is run. In this program, a background point is first obtained by monitoring with the excitation shutter closed and the emission shutter open. Then the sample with maximum fluorescence intensity (of those being studied) is inserted, both shutters are opened, and the sample

TABLE 1

Sample Protocol: quenching of +TG- by I- with Cl- as the ISA.

Solution #	ml Buffer 0.15M PO4 pH=6	ml Peptide +TG- A280=1	ml Quencher 5.0M NaI	ml ISA 2.5M NaCl	[NaI]	[NaCl]
1,1A	1.0	1.0	0.000	2.00	0.000	0.500
2	1.0	1.0	0.125	1.75	0.062	0.438
3	1.0	1.0	0.250	1.50	0.125	0.375
4	1.0	1.0	0.375	1.25	0.188	0.312
5	1.0	1.0	0.500	1.00	0.250	0.250
6	1.0	1.0	0.625	0.75	0.312	0.188
7	1.0	1.0	0.750	0.50	0.375	0.125
8	1.0	1.0	0.875	0.25	0.438	0.062
9	1.0	1.0	1.000	0.00	0.500	0.000

TABLE 2

Sample Protocol: quenching of +TG- by I- with F- as the ISA.

Solution #	ml Buffer 0.15M PO4 pH=6	ml Peptide +TG- A280=1	ml Quencher 5.0M NaI	ml ISA 0.625M NaF	[NaI]	[NaF]
1,1A	1.0	1.0	0.000	8.00	0.000	0.500
2	1.0	1.0	0.125	7.00	0.062	0.438
3	1.0	1.0	0.250	6.00	0.125	0.375
4	1.0	1.0	0.375	5.00	0.188	0.312
5	1.0	1.0	0.500	4.00	0.250	0.250
6	1.0	1.0	0.625	3.00	0.312	0.188
7	1.0	1.0	0.750	2.00	0.375	0.125
8	1.0	1.0	0.875	1.00	0.438	0.062
9	1.0	1.0	1.000	0.00	0.500	0.000

intensity is measured. This measurement is repeated a number of times and an average value is stored in the memory. This process is repeated for the remaining solutions.

After the data have been obtained, the computer is used for data manipulation. The program will print the intensity data, corrected for the sample sensitivity and background. The F_0/F ratio (where F_0 and F are the fluorescence intensities in the absence and presence of a given quencher) for the various concentrations of quencher in each solution is determined using this information.

C. A New Method for Determining F_0/F Ratios:

It was often difficult to obtain an accurate measurement of F_0 , the fluorescence intensity of a peptide solution in the absence of quencher, to be used in the calculation of F_0/F . This problem, due to fluctuations in the lamp intensity, caused large variations in F_0 measurements (as much as 7-10%) during the course of a single quenching experiment involving the measurement of the nine solutions with quencher concentration ranging from 0.0 to 0.5 molar. These variations in F_0 , and in the fluorescence intensities of the various solutions, make it very difficult to obtain reproducible data over a series of experiments.

In order to correct this problem a new procedure was

developed. In this procedure, three fluorescence cells are used, and relative fluorescence intensity measurements can be made on three samples very quickly (all three measurements can be made in about ten seconds). Solutions 1 and 1A, which contain no NaI quencher, are pipetted into two of the sample cells. A solution containing iodide quencher ranging in concentration from 0.062 to 0.500 molar is pipetted into the third cell (See Table 1 and Table 2 for protocol of solution preparation). Fluorescence intensities are then measured for the three solutions. The fluorescence intensity of solutions 1 and 1A are then averaged, and this value is F_0 . Solutions 1 and 1A are left in their cells, and the other solution is replaced in the third cell by another solution with iodide quencher. Again the fluorescence intensities of the three solutions are measured, and this procedure is repeated until all solutions containing iodide quencher are measured. Therefore, for each solution we have an intensity measurement for each solution containing varying quencher concentration and the measurement of the two solutions (1 and 1A) which contain no quencher; these three measurements are taken in a very short time period. For each solution of varying quencher concentration, the corresponding fluorescence intensities of solutions 1 and 1A are averaged, and this value is F_0 . The ratio of the value of F_0 (the first averaged value of 1 and 1A) to

each subsequent F_o' is used as a correctional factor in determining the fluorescence intensities of each of the solutions containing quencher. For example, suppose F_o equaled 235. If on a subsequent measurement, it is found that F_o' is 230, and the fluorescence intensity of the solution with quencher is 100, we can correct this quencher value by multiplying it by F_o/F_o' ($235/230$) giving a corrected value of 102.2 (F). F_o/F for each solution is then determined by dividing the value for F_o by the corrected fluorescence intensity, F (in this case $F_o/F = 235/102.2 = 2.30$).

This procedure, therefore, corrects for fluctuations in the lamp intensity which occur during a single experiment. The use of this procedure also means that differences in the fluorometer lamp intensity, not just during a single use, but on a day to day basis, will not affect the reproducibility of the data.

D. Quantum Yield Measurements:

The procedure outlined previously did not yield useful results when studying the anionic forms of the tryptophan containing peptides such as TGG-. The high basicity of the bicarbonate buffer (pH 9.8) may cause rapid decomposition of the peptides and create fluctuations in the data. However, the information needed from the anionic forms of the peptides could be obtained by making quantum yield measurements. The

solutions were prepared in a 10 ml volumetric flask and contained 1 ml bicarbonate buffer (pH 9.8), 1 ml peptide ($A_{280} = .8 - 1.2$), a 0.5 molar concentration of either NaCl or NaF, and the flask was diluted to the mark with dH₂O. Relative fluorescence measurements were made on a number of these solutions and the mean value of fluorescence intensity and standard deviation was determined for each type of solution (those with either 0.5 molar NaCl or with 0.5 molar NaF). The mean value for the fluorescence intensity of the solutions containing the NaF was normalized to a value of 1.00. The mean value for the fluorescence intensity of the solutions containing the NaCl was normalized to a relative value of that of the NaF solutions.

RESULTS

Looking at the data, we see a definite trend for those peptides containing a positive charge on the tryptophan residue (See Tables and Figures 3,5,7). There is a definite difference in the quenching (as much as 30 - 40 %) of these peptides by I⁻ depending on which of the two ISA's, NaCl or NaF, is used. The iodide quenching of these peptides when NaCl is the ISA is less than the quenching when NaF is the ISA. Clearly this means that the ISA's are having some kind of effect on the quenching when a positive charge is on the tryptophan. This effect is most noticeable for the peptide +TGG- (See Table and Figure 5) where the charge separation between the positive charge on the tryptophan and the negative charge on the glycine is the greatest in this study. The effect is still present, but reduced, as the charge separation is decreased. This can be seen in the results for the tryptophan (+T-, See Table and Figure 7) which contains both a positive and negative charge directly on the tryptophan. This implies that the negative charge on the tryptophan can not compensate for the effect when a positive charge is on the tryptophan.

In those peptides which contain a negatively charged or uncharged tryptophan, the iodide quenching is not reduced by the the use of NaCl rather than NaF as the ISA (See Tables and Figures 4,6,8). With a positive charge on the tryptophan, the iodide quenching with NaCl as the ISA was as much as 40% lower than the quenching with NaF as the ISA. However, with the negatively charged or uncharged tryptophan we notice that the iodide quenching is 2 to 6 % greater when NaCl is the ISA as opposed to NaF. This enhancement effect is most noticeable for the peptide NATA (See Table and Figure 8) which contains an uncharged tryptophan.⁽⁶⁾

The data in Table 9 show that this enhancement effect can also be seen for the quantum yield of an uncharged tryptophan such as that in anionic TGG-. The differences in the quantum yields for TGG- in the presence of NaCl and NaF was found to be statistically significant at a 95 - 99 % confidence level.

Table 10 shows the results of our fits to the modified Stern Volmer equation for each individual peptide. The Ksv value is the Stern Volmer quenching constant. These values allow for a direct comparison of the effect of the ISA's on each individual peptide. Note the reduced Ksv values for those peptides containing a positively charged tryptophan when NaCl is the ISA as opposed to NaF as the ISA. The second order quenching constant, k_q , can be determined by dividing Ksv by To

(the lifetime value in the absence of quencher). The k_q values reflect the collisional quenching of tryptophan by I⁻. Note that the k_q values are greater when a positive charge is on the tryptophan but that the magnitude of the differences for all the k_q values (whether the tryptophan is positively charged, negatively charged, or uncharged) fall within a factor of two.

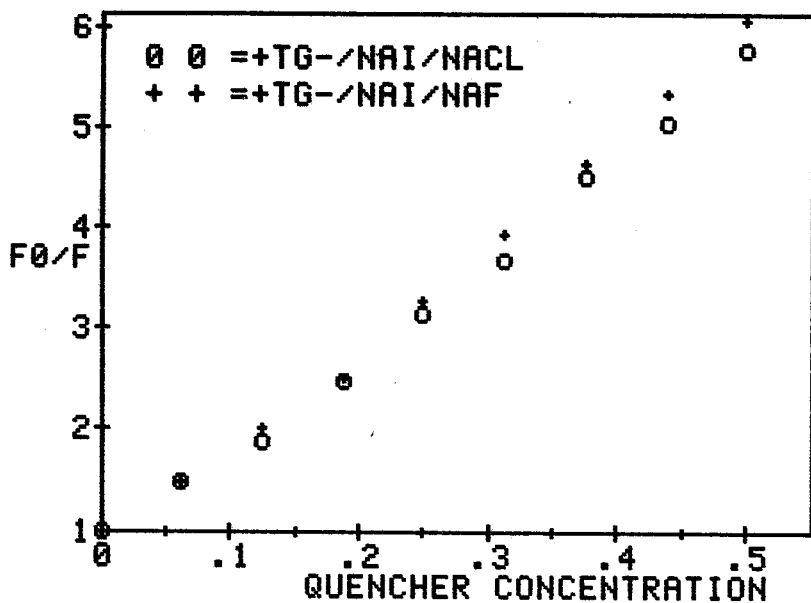
TABLE 3

Results for the quenching of +TC- by I- using Cl- or F- as the ISA.

Solution #	[NaI]	F ₀ /F Cl- ISA	Ave. Dev.	F ₀ /F F- ISA	Ave. Dev.
1	0.000	1.00	0.00	1.00	0.00
2	0.063	1.47	0.01	1.49	0.03
3	0.125	1.88	0.06	2.02	0.04
4	0.188	2.48	0.01	2.50	0.04
5	0.250	3.13	0.04	3.26	0.03
6	0.312	3.67	0.01	3.93	0.00
7	0.375	4.51	0.02	4.64	0.02
8	0.438	5.05	0.08	5.35	0.04
9	0.500	5.76	0.10	6.06	0.01

Temp = 21C Ex. = 295nm Em. = 348nm Ex. Slit = 6nm Em. Slit = 7nm

Figure 3



Stern Volmer plot for the quenching of +TG- by I- using Cl- or F- as the ISA.

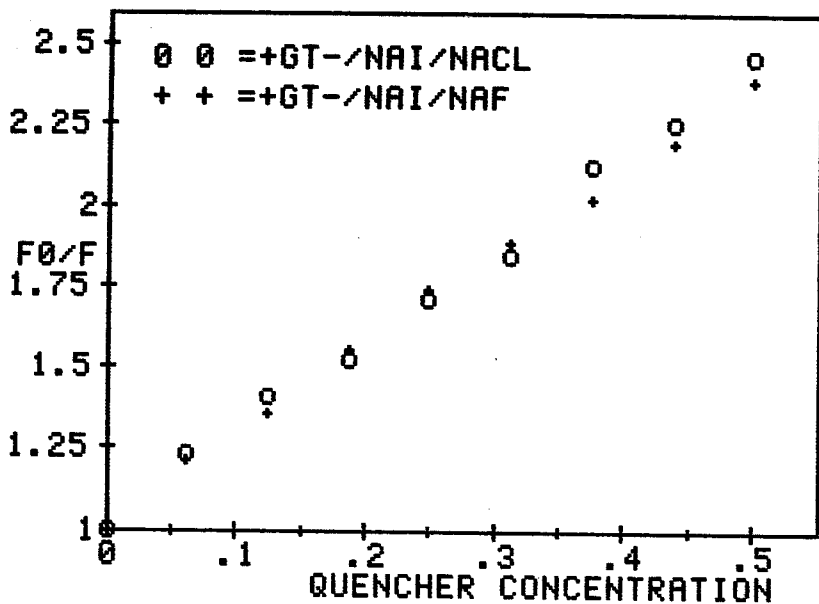
TABLE 4

Results for the quenching of +GT- by I- using Cl- or F- as the ISA.

Solution #	[NaI]	Fo/F Cl- ISA	Ave. Dev.	Fo/F F- ISA	Ave. Dev.
1	0.000	1.00	0.00	1.00	0.00
2	0.062	1.23	0.05	1.21	0.02
3	0.125	1.41	0.01	1.36	0.03
4	0.188	1.52	0.03	1.55	0.01
5	0.250	1.71	0.03	1.74	0.01
6	0.312	1.84	0.04	1.88	0.01
7	0.375	2.12	0.03	2.02	0.02
8	0.438	2.25	0.03	2.19	0.04
9	0.500	2.46	0.03	2.39	0.01

Temp = 21C Ex = 295nm Em. = 348nm Ex. Slit = 6nm Em. Slit = 7nm

Figure 4



Stern Volmer plot for the quenching of +GT- by I- using Cl- or F- as the ISA.

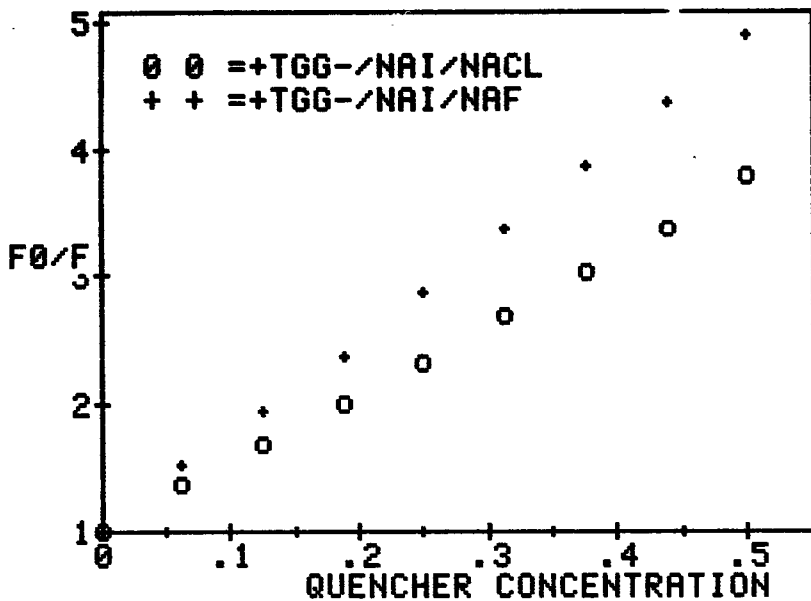
TABLE 5

Results for the quenching of +TGG- by I- using Cl- or F- as the ISA.

Solution #	[NaI]	Fo/F Cl- ISA	Ave. Dev.	Fo/F F- ISA	Ave. Dev.
1	0.000	1.00	0.00	1.00	0.00
2	0.062	1.35	0.01	1.51	0.00
3	0.125	1.68	0.01	1.94	0.02
4	0.188	1.98	0.02	2.36	0.03
5	0.250	2.32	0.00	2.86	0.02
6	0.312	2.69	0.02	3.38	0.04
7	0.375	3.02	0.01	3.88	0.02
8	0.438	3.38	0.01	4.36	0.07
9	0.500	3.78	0.03	4.90	0.02

Temp = 21C Ex. = 295nm Em. = 348nm Ex. Slit = 6nm Em. Slit = 7nm

Figure 5



Stern Volmer plot for the quenching of +TGG- by I- using Cl- or F- as the ISA.

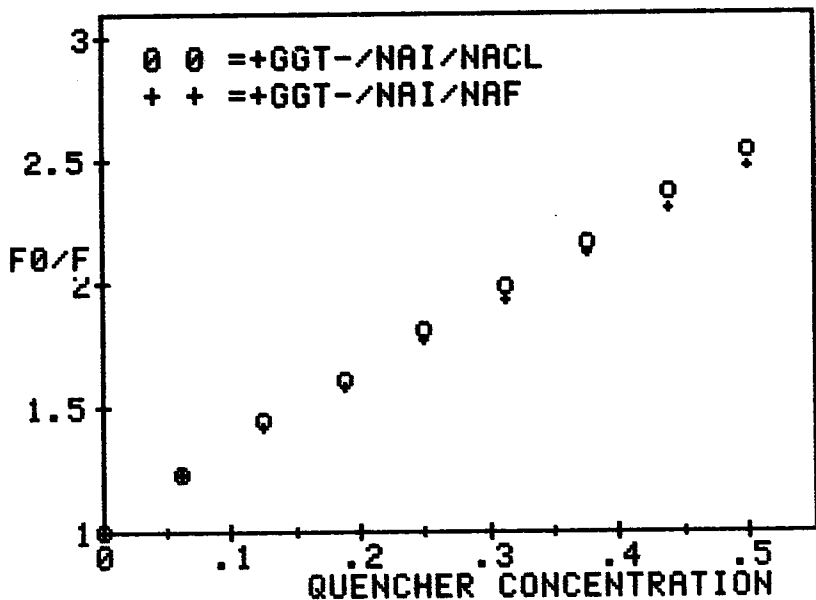
TABLE 6

Results for the quenching of +GGT- by I- using Cl- or F- as the ISA.

Solution #	[NaI]	Fo/F Cl- ISA	Ave. Dev.	Fo/F F- ISA	Ave. Dev.
1	0.000	1.00	0.00	1.00	0.00
2	0.062	1.23	0.00	1.23	0.01
3	0.125	1.44	0.02	1.41	0.01
4	0.188	1.60	0.02	1.57	0.01
5	0.250	1.80	0.02	1.76	0.02
6	0.312	1.98	0.02	1.93	0.00
7	0.375	2.16	0.02	2.12	0.01
8	0.438	2.36	0.01	2.30	0.02
9	0.500	2.54	0.02	2.47	0.00

Temp = 21C Ex. = 295nm Em. = 348nm Ex. Slit = 6nm Em. Slit = 7nm

Figure 6



Stern Volmer plot for the quenching of +GGT- by I- using Cl- or F- as the ISA.

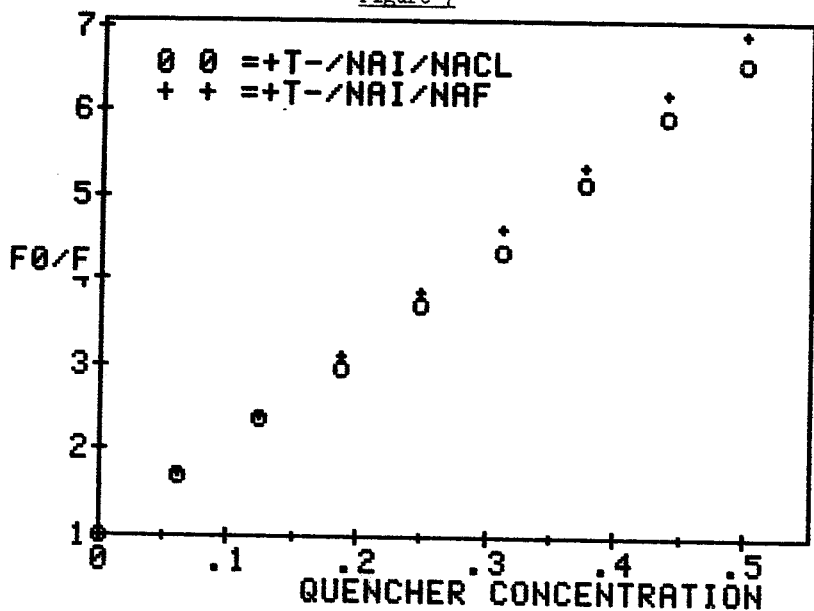
TABLE 7

Results for the quenching of Tryptophan by I- using Cl- or F- as the ISA.

Solution #	[NaI]	Fo/F Cl- ISA	Ave. Dev.	Fo/F F- ISA	Ave. Dev.
1	0.000	1.00	0.00	1.00	0.00
2	0.062	1.67	0.01	1.71	0.02
3	0.125	2.34	0.00	2.41	0.02
4	0.188	2.93	0.06	3.11	0.02
5	0.250	3.69	0.08	3.84	0.04
6	0.312	4.33	0.02	4.62	0.05
7	0.375	5.15	0.02	5.34	0.07
8	0.438	5.92	0.02	6.22	0.01
9	0.500	6.57	0.04	6.94	0.04

Temp = 21C Ex. = 295nm Em. = 348nm Ex. Slit = 6nm Em. Slit = 7nm

Figure 7



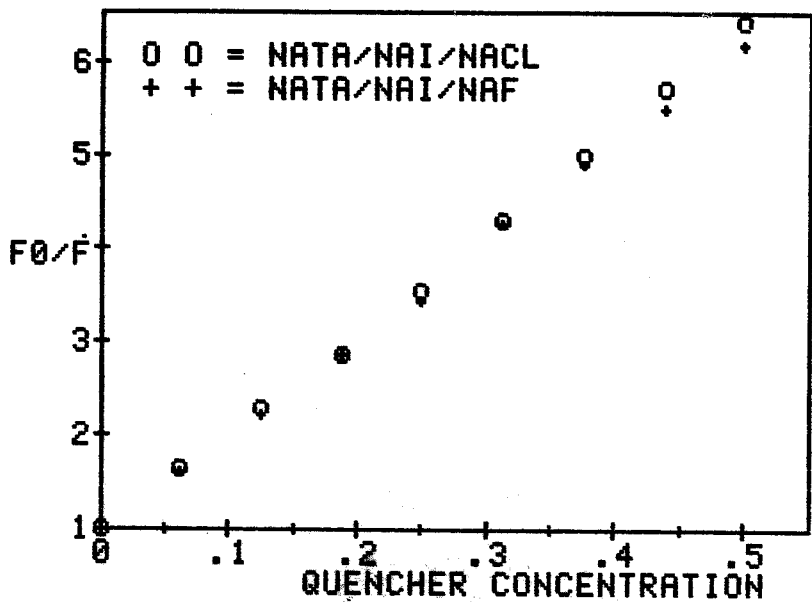
Stern Volmer plot for the quenching of tryptophan by I- using Cl- or F- as the ISA.

TABLE 8⁽⁶⁾

Results for the quenching of NATA by I- with Cl- or F- as the ISA.

<u>Solution #</u>	<u>[NaI]</u>	<u>Fo/F</u> <u>Cl- ISA</u>	<u>Fo/F</u> <u>F- ISA</u>
1	0.000	1.00	1.00
2	0.062	1.62	1.60
3	0.125	2.26	2.20
4	0.188	2.86	2.85
5	0.250	3.52	3.42
6	0.312	4.28	4.24
7	0.375	4.98	4.90
8	0.438	5.71	5.51
9	0.500	6.41	6.19

Figure 8



Stern Volmer plot for the quenching of NATA by I- using Cl- or F- as the ISA.

TABLE 9

Results for the relative quantum yield studies of the anionic peptide TGG-.

Peptide	[NaCl]	[NaF]	N	ϕ_a	s
TGG-	0.0	0.5	8	1.00	0.011
TGG-	0.5	0.0	7	1.02	0.012

(1) N = number of determinations.

The differences found in these quantum yields was found to be statistically significant at a 95-99% confidence level using the following t-test:

$$X1 - X2 = ts (N1 + N2)/N1N2$$

where t equals 2.16 at a 95% confidence level for the 13 degrees of freedom, s equals 0.012, and X1-X2 equals 0.02.

TABLE 10

Results of fitting data for each peptide to the modified Stern Volmer equation.⁽¹⁾

PEPTIDE	ISA = NaCl		ISA = NaF		$kq \times 10^{-9}$ ⁽³⁾
	K_{sv} ⁽²⁾	V	K_{sv}	V	
+TGG-	4.9	.18	7.0	.17	5.4
+TG-	7.1	.48	7.7	.46	4.6
+T-	10.0	.20	10.6	.20	4.0
+GGT-	3.1	0	3.0	0	3.2
+GT-	2.8	0	2.8	0	3.2
NATA	9.6	.20	9.3	.20	3.3

kq = rate constant for collisional quenching of the excited state

K_{sv} = Stern Volmer quenching constant

V = Static quenching constant

(1) $F_0/F_e^{(1)} = 1 + K_{sv}\langle Q \rangle$

(2) $K_{sv} = kq\tau_0$

(3) Units = $l \text{ mole}^{-1} \text{ sec}^{-1}$

DISCUSSION

Our secondary goal was to evaluate the role of commonly used ISA's such as NaCl in affecting the quenching of tryptophan containing peptides by I-. As stated in the results, Cl- had an inhibiting effect on the quenching of peptides containing a positively charged tryptophan residue. The inhibition of quenching can be seen by looking at the Ksv values of each particular peptide containing a positively charged tryptophan. In the case of +TGG-, where the charge separation is the greatest, the Ksv value when NaCl is the ISA is only 4.9 while the Ksv value when NaF is the ISA is 7.0. Similar, but lesser, effects are noticed for +TG- and +T-. We believe that the reason for this is that the Cl- is not acting as a benign ISA, but is actually quenching the fluorescence of tryptophan.

If Cl- quenches but F- does not, then the Fo value, which is the fluorescence of tryptophan in the absence of I- and in the presence of the maximum concentration of ISA, will be larger in the presence of F- where no quenching occurs than in the presence of Cl- where quenching occurs. If this is the case, then the ratio $F_o/F_e^{[q]}$ will be smaller in the presence of Cl- (See

Figure 9 for assistance in following this explanation).

The Cl^- therefore reduces the quenching by I^- .

Another way to look at what is occurring is to consider that if Cl^- is a quencher of tryptophan fluorescence, it will reduce the lifetime of the tryptophan. This means the excited states will exist a shorter time in the presence of Cl^- than they will exist in the presence of F^- . Because the excited states will not exist as long, I^- will not have as much time to quench the fluorescence of tryptophan, and, therefore, the amount of quenching by I^- will be less. Thus Cl^- reduces or inhibits I^- quenching of tryptophan.

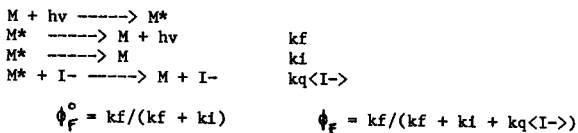
The Cl^- is not normally thought to be a quencher because it exhibits only a weak heavy atom attack. However, the positive charge on the tryptophan may draw the negatively charged Cl^- to it, allowing the Cl^- to remain in an encounter complex long enough to allow a quenching effect to be manifested.

By contrast, when a negative charge or no charge is on the tryptophan, as in $+\text{GGI}^-$, we find no inhibition of I^- quenching of these peptides in the presence of Cl^- . In these cases, there is no positive charge pulling the Cl^- to the tryptophan residue. In fact, there is a negative charge on the tryptophan repelling the negatively charged chloride. We do, however, notice a very small enhancement of iodide quenching of peptides containing a negatively or uncharged tryptophan when Cl^-

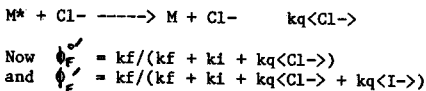
FIGURE 9

Explanation of how Cl^- reduces quenching by I^- .

For I^- quenching only:



With Cl^- present:



Thus: $\phi_F^0 > \phi_F'$ (if Cl^- quenching is measurable)

Furthermore: $\phi_F' \longrightarrow \phi_F$ as $\langle\text{Cl}^- \rangle \longrightarrow 0$

and at $\langle\text{Cl}^- \rangle = 0$ $\phi_F' = \phi_F$

Thus $\phi_F^0/\phi_F > \phi_F'/\phi_F$ as $\langle\text{Cl}^- \rangle \longrightarrow 0$

is present as the ISA. This effect can be seen by looking at the Ksv values for the peptide NATA which contains an uncharged tryptophan. The Ksv value in the presence of F- is only 9.3, but in the presence of Cl- is 9.6. We believe that the cause of this enhancement is due to a solvent effect. Water is the solvent for these studies and water is known to be a quencher of tryptophan (7) fluorescence. The Cl- might change the solvent enough so that the solvent quenches a small amount less than before and an enhancement of quenching results. The I- or Cl- in the presence of a positively charged tryptophan would also effect the solvent and cause this enhancement effect except that the quenching effect in both these cases is much greater than the enhancement effect and an overall quenching effect is witnessed.

In order to determine if this enhancement effect was actually occurring, quantum yield studies of another peptide with an uncharged tryptophan were performed. The peptide chosen was the anionic peptide TGG-. The tryptophan fluorescence in the presence of 0.5 molar Cl- exhibits only a 2% enhancement when compared to the fluorescence in the presence of 0.5 molar F-. However, this 2% enhancement is statistically significant at a 95 - 99 % confidence level.

Therefore, we see that Cl- quenches tryptophan fluorescence in peptides containing a positive charge on tryptophan and it slightly enhances tryptophan

fluorescence in peptides containing uncharged or negatively charged tryptophan residues. Since this is true, chloride containing salts are invalidated as ISA's.

Our main goal is to determine the magnitude of charge effects in the quenching of charged tryptophan containing peptides by I^- . In order to make this comparison, we compare the k_q values for all the peptides studied. Remember that k_q is the rate constant for the collisional deactivation of the excited state when a quencher is introduced into the system. We will compare the k_q values for the I^- quenching when NaF is the ISA, because in this system I^- is the only quencher. If the charge effect did not effect the quenching, we would expect all the k_q values to be the same because we would just have the iodide quenching of an accessible tryptophan in each case. However, when we look at the data, we see a definite difference between those cases where a positive charge is on the tryptophan pulling the I^- to it, as opposed to those cases where the tryptophan is uncharged or negatively charged. The k_q value is greatest for the peptide $+TGG^-$, where the separation between the positive and negative charge is the greatest. As the distance between the positive and negative charge decreases, as in $+TG^-$ or $+T^-$, the value of k_q decreases. However, these values are still greater than the values in the cases where there is a negative charge on the tryptophan, as in $+GGT^-$ or $+GT^-$, or no charge on the

tryptophan, as in NATA. The magnitude of these differences between the peptides with positively charged tryptophans and those with negatively charged or uncharged tryptophans is a factor of less than two. We conclude that the range of k_q values for exposed tryptophans in proteins should fall within a range of a factor of two.

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