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Triplet-Triplet Energy Transfer in Enzyme-Inhibitor Complexes

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TRIPLET-TRIPLET ENERGY TRANSFER IN ENZYME-INHIBITOR COMPLEXES

by

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INTRODUCTION

Previous investigations have demonstrated the presence of a tryptophan residue in the active site of carbonic anhydrase by using an inhibitor as a phosphorescent probe (1). The inhibitor possessed a lowest triplet state which was more energetic than that of tryptophan, and observation of triplet-triplet energy transfer indicated that tryptophan was at the active site. The purpose of the present research was to explore the usefulness of the inhibitor probe technique by investigating other inhibitors having diverse spectral properties.

In a phosphorescent probe, intermolecular triplet-triplet energy transfer is utilized, both the theory and practice of which are well documented (2,3,4). It has been demonstrated that this type of transfer is a short range process, 15 A or less (5). The most probable mechanism for this type of transfer involves electron transfer via overlap of molecular orbitals (6).

A competing process may be intermolecular singlet-singlet energy transfer, which can occur at distances up to 50 A, probably via an induced dipole mechanism (7). This type of transfer may be ruled out or differentiated as will be seen. Singlet-triplet and triplet-singlet transfer may be ruled out on the basis of quantum mechanical selection rules.

Carbonic anhydrase is a metallo-enzyme containing one molecule of zinc covalently bound in the active site and 25 aromatic amino acid residues, seven of which are tryptophan (8). The phosphorescence

spectrum of carbonic anhydrase is exclusively that of tryptophan owing to facile energy transfer to tryptophan from the other aromatic amino acids (9).

It has been shown that sulfonamides inhibit carbonic anhydrase by binding to the zinc in the active site of the enzyme, and that one molecule of sulfonamide binds to one molecule of enzyme (10). Therefore, if there is triplet-triplet energy transfer between a bound inhibitor molecule and a tryptophan, it can be assumed that the tryptophan must also be in the active site due to the short range nature of this process.

There is a possibility of energy transfer to a tyrosine which then transfers to an adjacent tryptophan via a chain type mechanism. This can be ruled out by the proper choice of inhibitor energy levels as will be seen.

Since triplet-triplet energy transfer experiments are performed at 77°K, it is essential that no gross conformational change in the enzyme occurs upon freezing. This could give rise to transfer to or from a tryptophan that was originally much farther away in the native enzyme. It has been recently reported that a variety of synthetic and natural proteins frozen quickly by immersion in liquid nitrogen showed no conformational changes (11,12). This indicates that on quick freezing one would expect no gross conformational change in carbonic anhydrase.

EXPERIMENTAL

Materials

Bovine erythrocyte carbonic anhydrase (3125 EU/mg.) was obtained from Worthington and used without further purification. Ethylene glycol and sodium borate decahydrate were Baker "Reagent" grade. The ethylene glycol was further purified by fractionation through a twelve inch column packed with three millimeter glass helices.

Acetazolamide was purchased from K and K Chemicals and 5-ethoxy-2-benzothiazolesulfonamide was a gift of the Upjohn Co. 3-Acetylbenzenesulfonamide, 4-bromobenzenesulfonamide, and 4,4'-biphenyldisulfonamide were purchased from Aldrich as sulfonyl halides and converted to sulfonamides by ammonolysis. The remaining sulfonamides were synthesized via standard methods (13,14,15). All of these compounds were recrystallized twice from 1/1 (v/v) ethanol/water and gave standard melting points.

DEAE Sephadex A50 anion exchange resin was purchased from Pharmacia. Tris(hydroxymethyl)aminomethane (Tris) was Sigma "Primary Standard" grade.

Apparatus

All absorption spectra were obtained at room temperature using a Cary 14 recording spectrophotometer. The emission spectra were obtained in a rigid solvent glass at 77°K using an Aminco-Bowman Spectrophotofluorometer equipped with an ellipsoidal condensing mirror system and a Hewlett-Packard Moseley 7035A X-Y recorder.

Specially made quartz dewar flasks were obtained to accomodate seven millimeter I.D. quartz sample tubes. A rotating cylindrical shutter was constructed to accomodate the quartz dewar flasks. With this shutter it was possible to have two slits 180° to each other, or four slits 90° to each other. This permitted the measurement of both phosphorescence, using the first slit arrangement, and fluorescence, using the second slit arrangement, at 77°K and higher temperatures.

A six liter Thermos dewar flask was equipped with nichrome heating element and a variable transformer to permit the generation of cold nitrogen gas for temperature dependence studies. The cold nitrogen gas thus generated was routed via tubing into the quartz sample flask. This allowed studies in the temperature range from 100°K to 200°K.

The temperature of the sample was monitored using a chromel-alumel thermocouple immersed in the sample. The millivolt readings were made with a Honeywell Electronik 194 recorder.

Methods

Relative amounts of isoenzymes were determined by the method of Armstrong (16). This involves elution with 0.05 M Tris buffer (pH 8.7 at 20°C) through a DEAE Sephadex A50 column. Isoenzymes A, B, and C may also be separated in this manner.

All solutions were made up in 1/1 (v/v) ethylene glycol/0.01 M aqueous sodium borate buffer (pH 9.18 at 20°C). Inhibitor solutions were 5.0×10^{-4} M and carbonic anhydrase solutions were 5.5×10^{-4} M.

Standard carbonic anhydrase/inhibitor solutions had final concentrations of $5.0X10^{-4}$ M/$5.0X10^{-4}$ M so that excess enzyme was always present. The reproducibility of phosphorescent intensity of this type type of glass at 77°K was $\pm$ 10%.

Dissociation constants (K_I) for the enzyme-inhibitor complexes were determined by the method of Wilbur and Anderson (17) using a Beckman Model H2 pH meter with combination electrode. Enzymic activity was determined similarly.

Estimates of the 0-0 singlet and triplet energy levels were made for each inhibitor. Proceeding from shorter to longer wavelengths through a particular spectrum, the wavelength of absorption cut-off and phosphorescence onset were used to calculate the lowest singlet and triplet levels, respectively. Approximate ($\pm$ 0.2 seconds) phosphorescent lifetimes, the time required for decay to 1/e of the original intensity, were determined by interrupting the exciting light and recording the decay time with an electronic timer.

In some cases it was necessary to observe the phosphorescence spectrum 5.0 seconds after the exciting light had been interrupted. This was accomplished by interrupting the exciting light, waiting 5.0 seconds, and recording the phosphorescent intensity at a given wavelength. By repeating this process at five nm. intervals it is possible to construct the phosphorescence spectrum remaining after 5.0 seconds.

A phototube calibration curve was determined by comparing fluorescence spectra obtained using our instrument with corrected spectra for the same compounds (18), a method which is good to an

accuracy of ±5%. To obtain quantum efficiencies of fluorescence (ϕ_F) and phosphorescence (ϕ_P), our manually corrected spectra were graphically integrated. These values were then used in a standard quantum efficiency formula (19). The quantum efficiencies thus obtained are accurate to ± 15% from the combination of glass reproducibility and phototube calibration.

Filters used were a 295 nm. sharp cut-off filter (Oriel) and a one millimeter quartz cell containing a 0.2 M aqueous solution of the sodium salt of tryptophan. This latter filter gave a 313 nm. cut-off and allowed no excitation of the aromatic amino acid residues in carbonic anhydrase.

THEORY

If tryptophan is considered as one of the chromophores, and the bound inhibitor as the second chromophore, there are four possible permutations of energy levels. This is illustrated in figure 1.

In case 1 it is possible to excite only the inhibitor by using the tryptophan filter mentioned earlier. The excited inhibitor may decay in energy to its triplet state via intersystem crossing. The inhibitor triplet may now emit a quantum of light as phosphorescence or, under favorable conditions, undergo triplet-triplet transfer with an adjacent chromophore, in this case a tryptophan. If there is complete transfer one would observe only a tryptophan phosphorescence spectrum with its characteristic long lifetime, 5.7 seconds.

If there is incomplete transfer one would observe a combination spectrum of the inhibitor and tryptophan which would have a short-lived component, the inhibitor phosphorescence, and a long-lived component, the tryptophan phosphorescence. This may be dealt with experimentally if the inhibitor has a short-lived phosphorescence by allowing a time corresponding to several inhibitor phosphorescent lifetimes to pass and recording the phosphorescent intensity at a given wavelength. By doing this manually from 380 nm. to 550 nm. and plotting the resultant intensities, one would obtain a tryptophan phosphorescence spectrum if there was actually intermolecular triplet-triplet energy transfer.

As further evidence of triplet-triplet energy transfer it should be possible to eliminate or reduce this long-lived component by dis-

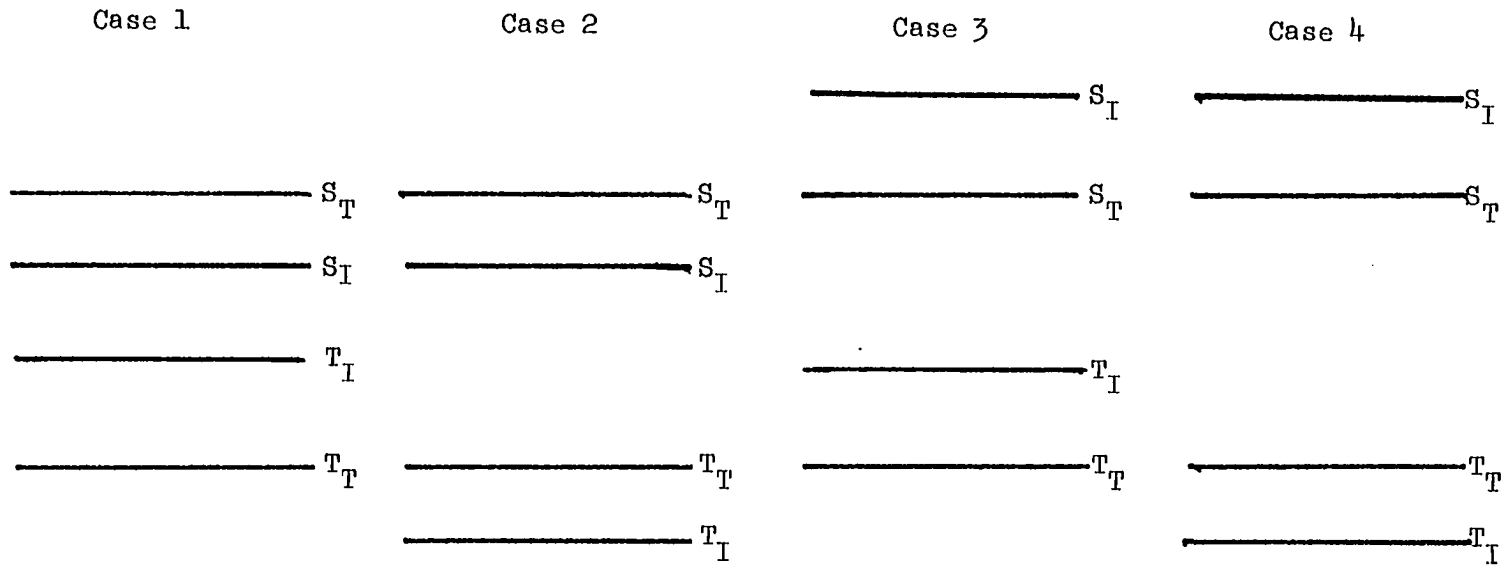

Figure 1. Permutations of inhibitor singlet (S_I) and triplet (T_I) levels with tryptophan singlet (S_T) and triplet (T_T) levels relative to the ground state.

placing all or most of the phosphorescing inhibitor with a stronger non-phosphorescing inhibitor. Depending on concentrations and K_I's, either complete or partial remission of tryptophan phosphorescence would occur.

Case 2 must be approached differently. There are two ways of treating case 2 compounds, the first of which is similar to case 1 compounds.

In the first method only the inhibitor may be excited as previously described. Via intersystem crossing it may decay to its triplet state, but triplet-triplet energy transfer is energetically unfavorable as it is an endergonic process at 77°K. By warming the sample it may now be possible with this added thermal excitation to induce transfer to an adjacent tryptophan. It has been demonstrated that at 150°K this thermally induced transfer should occur if 2000 cm^{-1} or less separate the two triplet states (20). Since this type of transfer is kinetically unfavorable there must also be some type of trapping mechanism at the higher level. If this type of transfer occurred, characteristic tryptophan phosphorescence of shorter wavelength would appear. With transfer, a combination spectrum would result with shoulders in the vicinity of normal tryptophan peaks.

A possible artifact in this type of study would be thermally induced singlet-singlet transfer from the inhibitor to the tryptophan. By observing both fluorescence and phosphorescence at the elevated temperature the presence of singlet-singlet transfer could be determined. If such transfer occurred one would observe tryptophan fluorescence as well as phosphorescence. Also large differences

in singlet level energies would preclude the possibility of transfer.

Another way of dealing with case 2 compounds is to choose a wavelength of light that will be absorbed by both the enzyme and the inhibitor. In this case, if triplet-triplet transfer was to occur one would expect the energetically favorable transfer from tryptophan to the inhibitor as can be seen from figure 1. This then implies a decrease in tryptophan phosphorescence with an increase in inhibitor phosphorescence. With transfer a combination spectrum would be obtained.

An important artifact to be considered here is singlet-singlet transfer from tryptophan to the bound inhibitor molecule. If singlet-singlet transfer occurs, the fluorescence of tryptophan will be decreased but the tryptophan phosphorescence/fluorescence intensity (P/F) ratio will remain unchanged as phosphorescence is directly proportional to the fraction of occupied singlets. If triplet-triplet transfer is occurring, the P/F ratio for tryptophan will decrease since the occupied tryptophan triplet state is depleted by transfer. Therefore, a decreased tryptophan P/F ratio implies triplet-triplet transfer is occurring rather than singlet-singlet transfer. Along with this quenching of tryptophan phosphorescence by triplet-triplet transfer, one would expect a significantly shorter lifetime as inhibitor phosphorescence is enhanced.

Case 3 may also be studied in two ways. In the first method tryptophan alone may be excited by the proper choice of cut-off filter. The tryptophan may decay to its triplet state by intersystem crossing. By thermal excitation, as in case 2, it may be

possible to observe triplet-triplet transfer from tryptophan to the bound inhibitor molecule. If this occurred one would observe characteristic inhibitor phosphorescence at shorter wavelengths. As before, singlet-singlet transfer from the thermally excited tryptophan would give characteristic inhibitor fluorescence as well as phosphorescence.

Another method of studying case 3 would be to excite both the tryptophan and the inhibitor. In this case, the energetically preferred pathway would be triplet-triplet transfer from the bound inhibitor to the tryptophan as can be seen from figure 1. If this occurred one would observe a quenching of the inhibitor phosphorescence and an enhancement of tryptophan phosphorescence. This would give rise to an increased P/F ratio for tryptophan. The lifetime would be expected to approach that of tryptophan if transfer is efficient.

As with case 2, singlet-singlet transfer from the inhibitor to tryptophan may also occur. This would result in no change in the tryptophan P/F ratio as both tryptophan fluorescence and phosphorescence would be increased by the same factor.

Case 4 compounds may be studied in much the same way as case 1 compounds. With the proper filter only tryptophan is excited. The tryptophan may decay to its triplet state and via triplet-triplet transfer induce phosphorescence from the inhibitor which would appear at a longer wavelength than tryptophan. Unfortunately no case 4 chromophores were found. This was not unexpected since very few compounds with larger singlet-triplet splitting than tryptophan are known.

RESULTS

General

By elution through a DEAE Sephadex A50 column with 0.05 M Tris buffer (pH 8.7 at 20°C) it was found that the carbonic anhydrase used in this study was a mixture of isoenzymes A, B, and C. It was found that the ratio B/A/C was about 18/1/1. Since the purpose of this research was to expand a novel technique, it wasn't deemed necessary to use a pure isoenzyme. As long as at least one component of the mixture had a tryptophan in its active site, energy transfer could be observed.

It was found that a standard solution of the enzyme frozen quickly by immersion in liquid nitrogen showed no change in activity on thawing. This lends support to the assumption of no gross conformational changes on quick freezing.

A minor phosphorescing impurity was found to be present in the enzyme. Its phosphorescence peak appears only in high sensitivity spectra and does not interfere appreciably, due to its short phosphorescent lifetime and wavelength of emission maximum.

From the order of magnitude of the K_I's for the various enzyme-inhibitor complexes, shown in table 1, it can be said that there is very little dissociation of the complex once it is formed. Therefore, since excess enzyme was used there should be very little, if any, free inhibitor in the enzyme/inhibitor solutions. This eliminates the possibility of transfer between an unbound inhibitor molecule and a tryptophan not in the active site of the enzyme.

Table 1. General Properties of the Inhibitors

Inhibitor	Absorption Cut-off (nm)	Phosphorescence Onset (nm)	ϕ_P	ϕ_F	K_I (M)
I	340	350	0.12	--	1.0×10^{-6}
II	320	355	0.22	0.070	9.0×10^{-7}
III	345	365	0.47	0.014	1.3×10^{-7}
IV	345	390	0.21	0.029	1.8×10^{-8}
V	335	440	0.0027	0.055	1.0×10^{-7}
VI	290	355	0.18	--	1.7×10^{-7}
VII	292	342	0.081	0.0078	1.7×10^{-6}
VIII	288	340	0.16	--	6.3×10^{-6}
IX	310	365	0.10	0.088	9.8×10^{-6}
X	283	350	0.063	--	4.7×10^{-8}
XI	290	360	0.092	--	4.4×10^{-8}

Following are the results with the inhibitors. All case 1 inhibitors exhibited triplet-triplet energy transfer.

Case 1 Inhibitors

3-Acetylbenzenesulfonamide

The spectra of 3-acetylbenzenesulfonamide (I) are shown in figure 4. The phosphorescent lifetime is less than one second. This is the inhibitor originally used by Galley and Stryer to demonstrate the presence of a tryptophan residue in the active site of carbonic anhydrase.

When a standard solution of I and carbonic anhydrase was excited at 320 nm. using the tryptophan filter, a phosphorescence spectrum illustrated as the top curve in figure 5 is obtained. This is the combined emission of I, tryptophan, and an enzyme impurity peak at 480 nm. This enzyme impurity peak showed up only at high sensitivity settings and in no way interferred with energy transfer.

The spectrum illustrated as the bottom curve in figure 5 was obtained by plotting phosphorescent intensity 5.0 seconds after the excitation beam had been interrupted. This spectrum matches that of tryptophan in figure 2 and has the 5.6 second characteristic lifetime of tryptophan.

When a standard solution of I with carbonic anhydrase and a 300% excess of acetazolamide (K_I = 6 X 10^{-9}M) was excited under the same conditions, the tryptophan shoulders at 415nm. and 440nm. disappeared. There was also no long-lived component of emission.

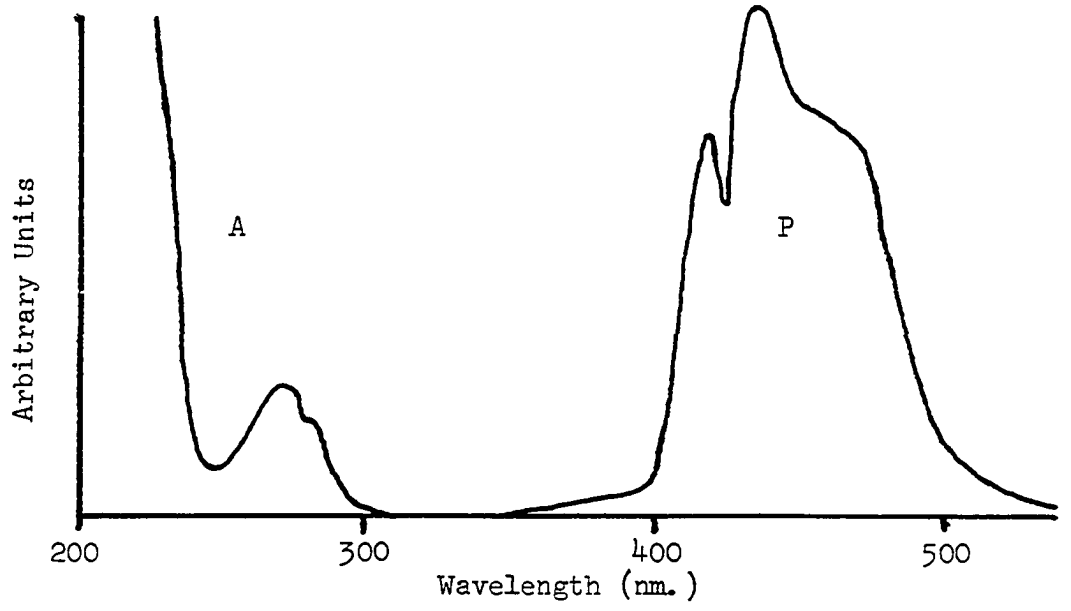

Figure 2. Absorption (A) and phosphorescence (P) spectra of carbonic anhydrase. P was excited at 250 nm.

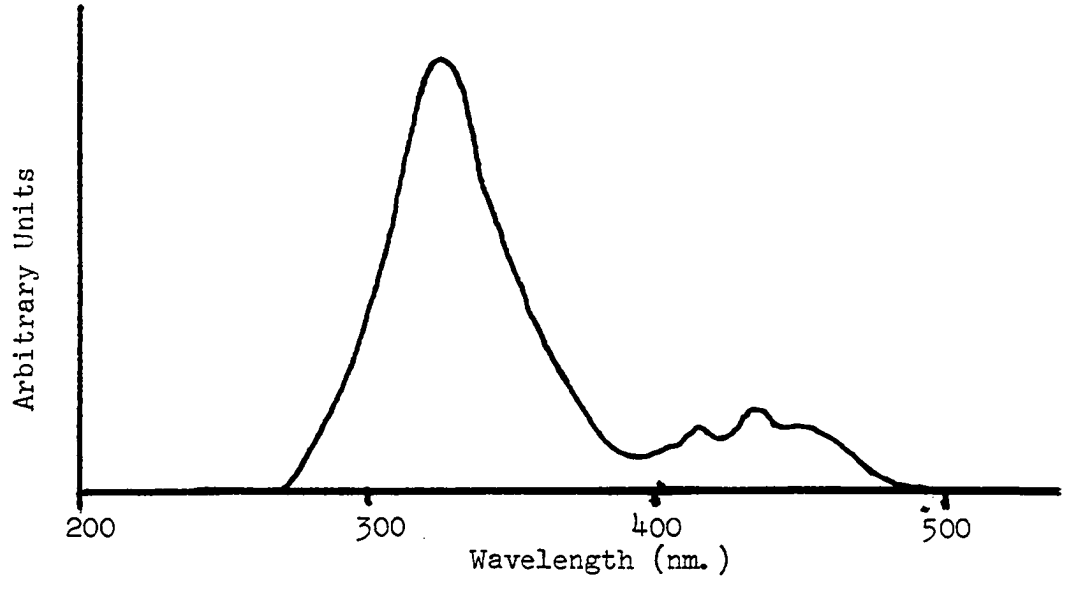

Figure 3. Phosphorescence/fluorescence spectrum of carbonic anhydrase excited at 250 nm.

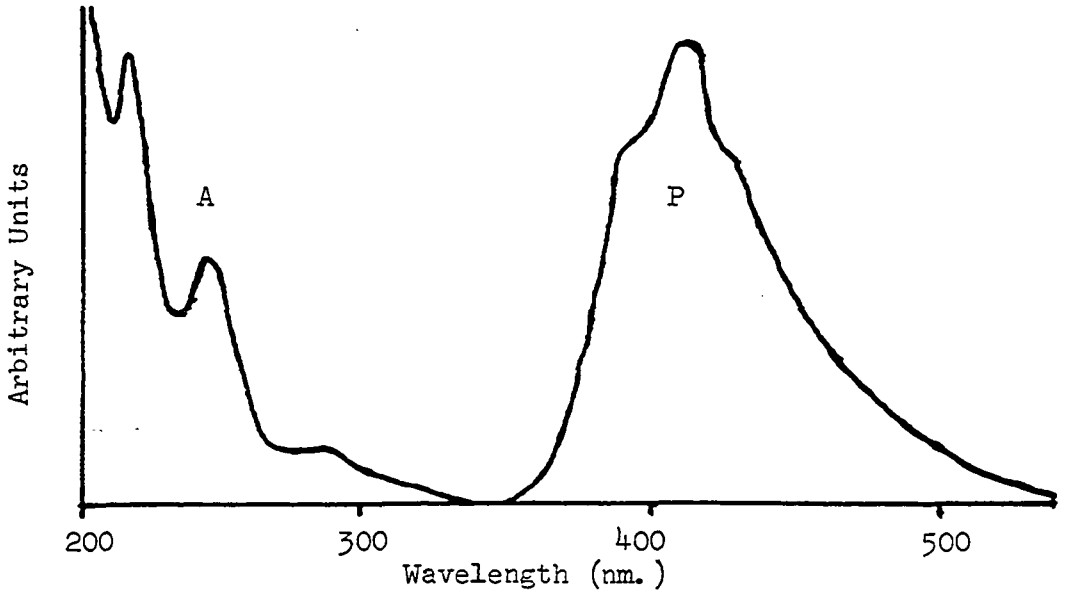

Figure 4. Absorption (A) and phosphorescence (P) spectra of 3-acetylbenzenesulfonamide. P was excited at 320 nm.

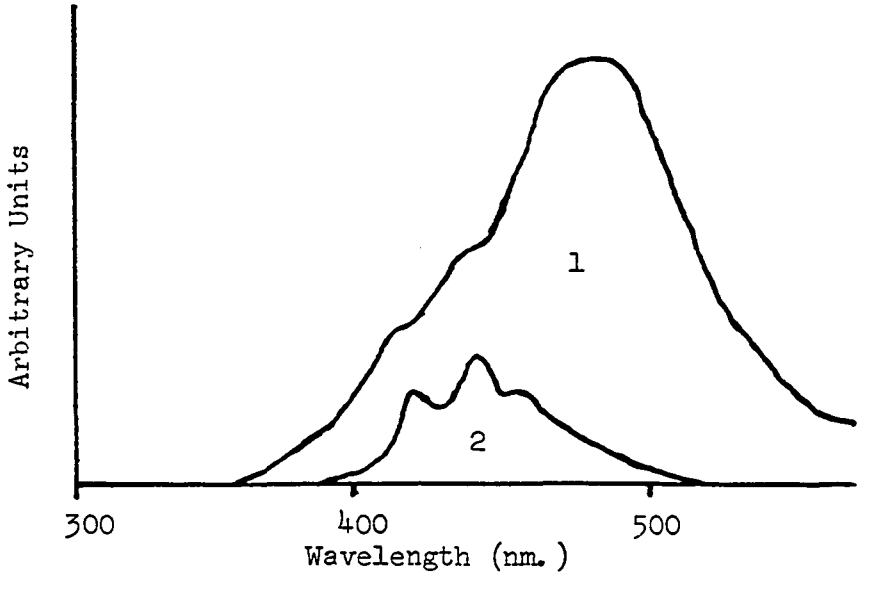

Figure 5. Emission spectra of a standard solution of 3-acetylbenzenesulfonamide and carbonic anhydrase excited at 320 nm. 1-initially. 2-after 5.0 seconds.

The data indicate that there was triplet-triplet energy transfer from I to a tryptophan in the active site of carbonic anhydrase. The disappearance of transfer with excess acetazolamide is expected, since it is a stronger inhibitor than I by a factor of about 1000.

4-Aminobenzenesulfonamide

The spectra of 4-aminobenzenesulfonamide (II) are shown in figure 6. This inhibitor has a phosphorescent lifetime of less than one second.

When a standard solution of II and carbonic anhydrase was excited at 320 nm. using a tryptophan filter, the phosphorescence spectrum corresponding to the upper curve in figure 7 was obtained. This appears to be a combination of the phosphorescence of II and the enzyme impurity.

The phosphorescent intensity 5.0 seconds after excitation is shown as the bottom curve in figure 7. The band shape and 5.6 second lifetime are characteristic of tryptophan.

When a standard solution of II with carbonic anhydrase and a 300% excess of acetazolamide was excited under the same conditions, the long-lived component of emission disappeared. After 5.0 seconds there was no detectable phosphorescence from this sample. Complete disappearance of this transfer is expected in the presence of excess acetazolamide, since it is a stronger inhibitor than II by a factor of about 1000.

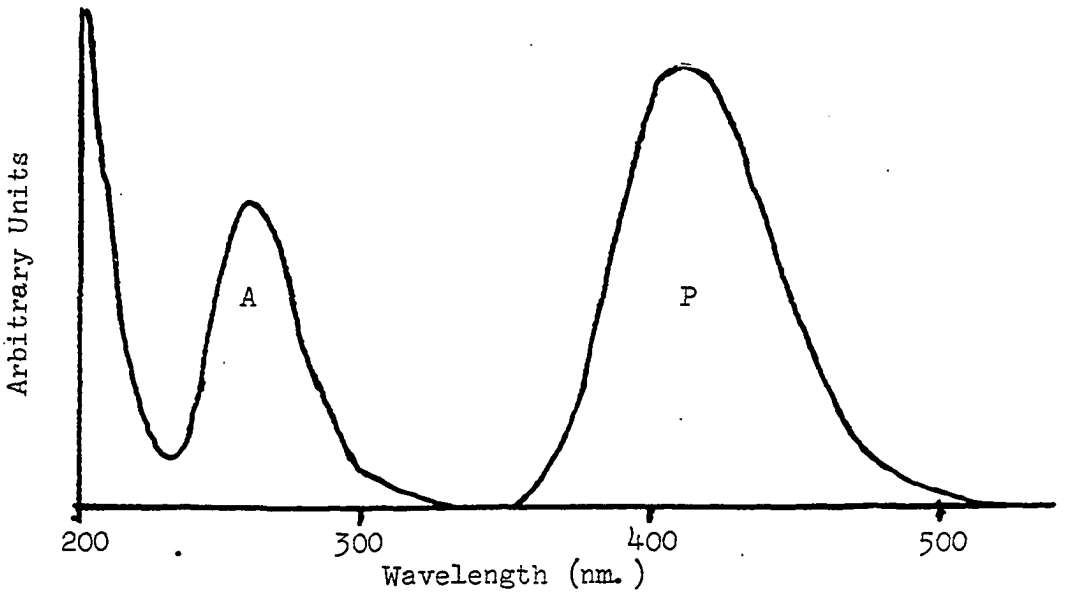

Figure 6. Absorption (A) and phosphorescence (P) spectra of 4-aminobenzenesulfonamide. P was excited at 320 nm.

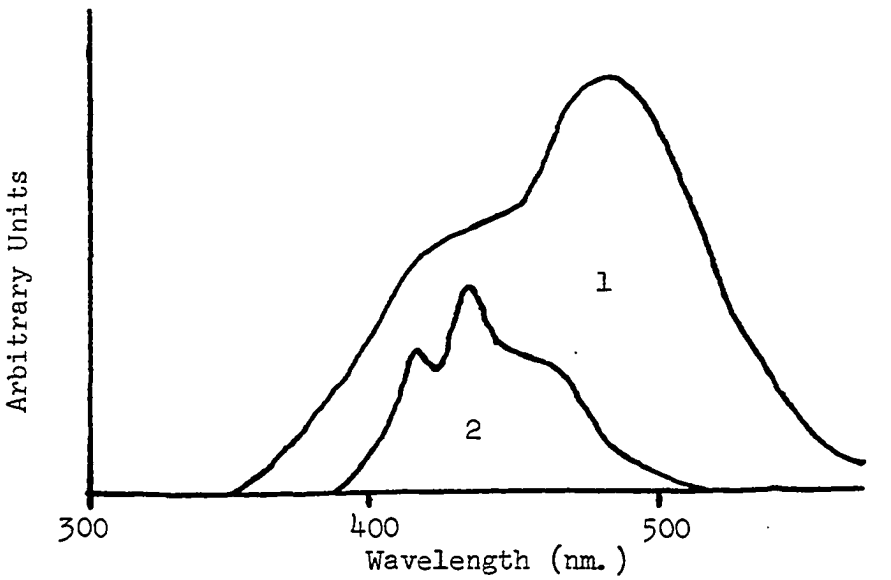

Figure 7. Phosphorescence spectra of a standard solution of 4-aminobenzenesulfonamide and carbonic anhydrase excited at 320 nm. 1-initially. 2-after 5.0 seconds.

2-Amino-4-chlorobenzenesulfonamide

The spectra of 2-amino-4-chlorobenzenesulfonamide (III) are shown in figure 8. This inhibitor has a phosphorescent lifetime of less than one second.

When a standard solution of III and carbonic anhydrase was excited at 320 nm. using a tryptophan filter, the phosphorescence spectrum illustrated as the upper curve in figure 9 resulted. This appears to be solely an inhibitor spectrum, but was observed to contain a small long-lived component.

The phosphorescent intensity 5.0 seconds after excitation is shown as the bottom curve in figure 9. The band shape and 5.6 second lifetime are characteristic of tryptophan.

When a standard solution of III with carbonic anhydrase and a 300% excess of acetazolamide was excited under the same conditions, the long-lived component was decreased to about 10% of its value in the absence of acetazolamide. A plot of phosphorescent intensity after 5.0 seconds yielded a tryptophan spectrum that was similarly reduced in magnitude. The lack of total remission of tryptophan phosphorescence is not unexpected as acetazolamide is a stronger inhibitor than III by only a factor of about 100.

3-Amino-4-chlorobenzenesulfonamide

The spectra of 3-amino-4-chlorobenzenesulfonamide (IV) are shown in figure 10. This inhibitor has a phosphorescent lifetime of less than one second.

When a standard solution of IV and carbonic anhydrase was ex-

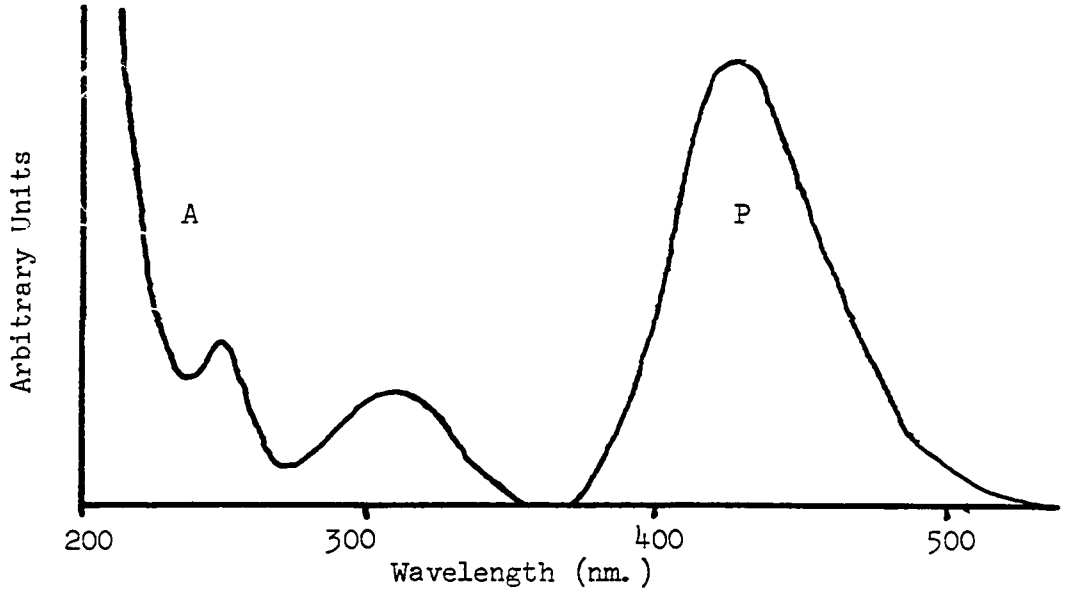

Figure 8. Absorption (A) and phosphorescence (P) spectra of 2-amino-4-chlorobenzenesulfonamide. P was excited at 320 nm.

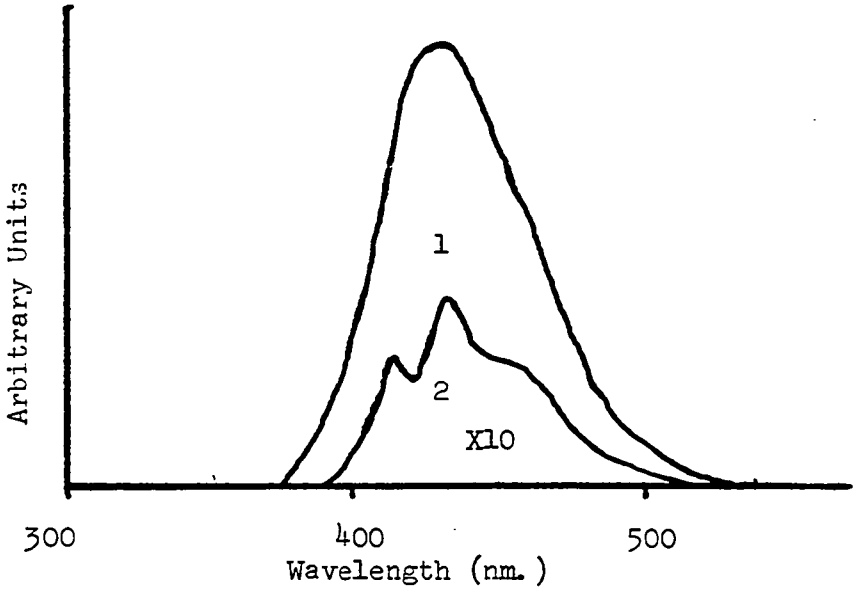

Figure 9. Phosphorescence spectra of a standard solution of 2-amino-4-chlorobenzenesulfonamide and carbonic anhydrase excited at 320 nm. 1-initially. 2-after 5.0 seconds.

cited at 320 nm. using a tryptophan filter, the phosphorescence spectrum illustrated as the upper curve in figure 11 was obtained. This appears to be solely an inhibitor phosphorescence spectrum but was observed to contain a small long-lived component.

The phosphorescent intensity 5.0 seconds after excitation is shown as the bottom curve in figure 11. The band shape and 5.7 second lifetime are characteristic of tryptophan.

When a standard solution of IV with carbonic anhydrase and a 300% excess of acetazolamide was excited under the same conditions, the long-lived component of emission was reduced to about 20% of its value in the absence of acetazolamide. A plot of phosphorescent intensity 5.0 seconds after excitation yielded a tryptophan spectrum that was similarly reduced in magnitude. The lack of total remission of tryptophan phosphorescence is not unexpected, as acetazolamide is a stronger inhibitor than IV by only a factor of about 10.

Case 2 Inhibitors

The spectra of 2-napthalenesulfonamide (V) are shown in figure 12. This inhibitor has a phosphorescent lifetime of 1.7 seconds.

When a standard solution of V and carbonic anhydrase was excited at 320 nm. using a tryptophan filter, only an inhibitor phosphorescence spectrum resulted. Under the same conditions it was found that in the temperature range from 77°K to 160°K only an inhibitor phosphorescence spectrum could be obtained. Therefore, it was not possible to thermally induce triplet-triplet transfer from V to a tryptophan in the active site of carbonic anhydrase in this

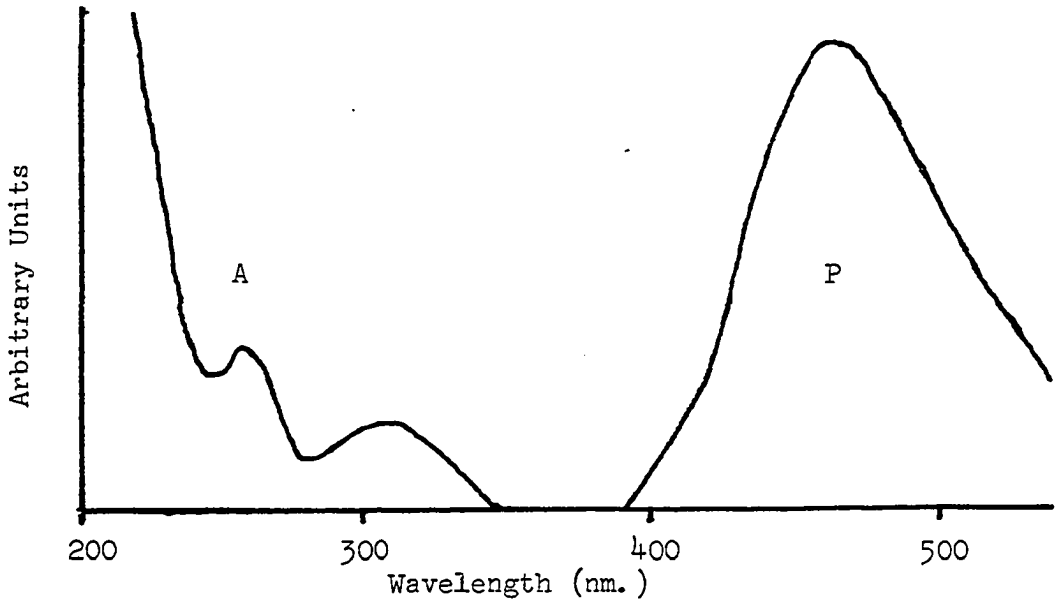

Figure 10. Absorption (A) and phosphorescence (P) spectra of 3-amino-4-chlorobenzenesulfonamide. P was excited at 320 nm.

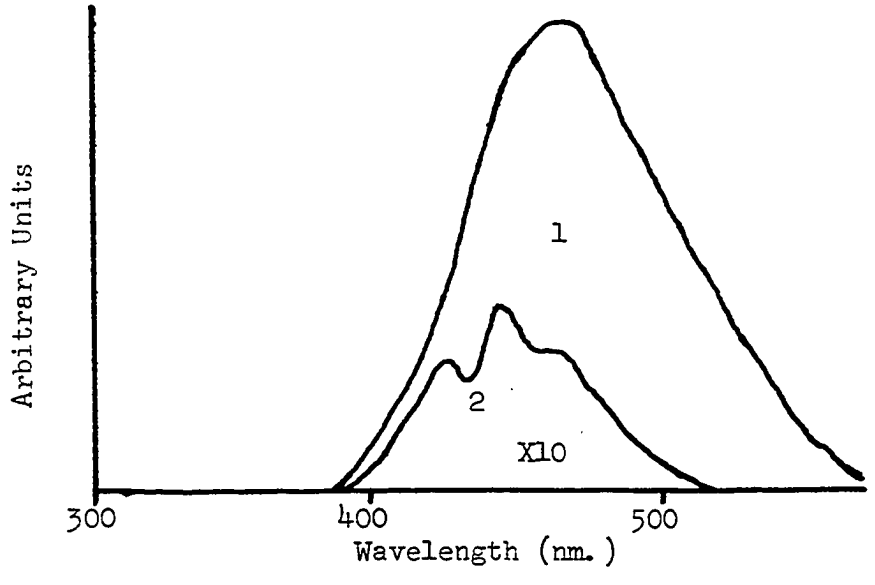

Figure 11. Phosphorescence spectra of a standard solution of 3-amino-4-chlorobenzenesulfonamide and carbonic anhydrase excited at 320 nm. 1-initially. 2-after 5.0 seconds.

temperature range. Identical results were obtained with all case 2 inhibitors tested.

When a standard solution of V and carbonic anhydrase was excited at 250 nm., the phosphorescence spectrum illustrated in figure 13 was obtained. Considering the relative absorbanices of V and the enzyme at 250 nm., it was found that the tryptophan phosphorescence was about 40% quenched. This implies that either the singlet or triplet state of tryptophan is being depleted by energy transfer.

Previous studies on standard solutions of carbonic anhydrase revealed that the P/F ratio for tryptophan in the enzyme excited at 250 nm. was 0.15, as illustrated in figure 3. The P/F ratio for tryptophan in standard solutions of V and carbonic anhydrase excited at 250 nm. was found to be 0.12 and 0.12 in duplicate experiments. It was also demonstrated that only the phosphorescence of tryptophan was decreased; the expected amount of tryptophan fluorescence was obtained.

The data demonstrate that only the triplet state of tryptophan was depleted, and therefore, triplet-triplet transfer from a tryptophan in the active site of the enzyme to V occurred.

2-Chloro-5-aminobenzenesulfonamide, 8-quinolinesulfonamide, 4,4'-biphenyldisulfonamide, and 5-ethoxy-2-benzothiazolesulfonamide are all case 2 inhibitors. Of these, the first three exhibited no transfer, either singlet-singlet or triplet-triplet.

With 5-ethoxy-2-benzothiazolesulfonamide singlet-singlet transfer from tryptophan was demonstrated. The fluorescence and phosphorescence of tryptophan were both quenched relative to the fraction

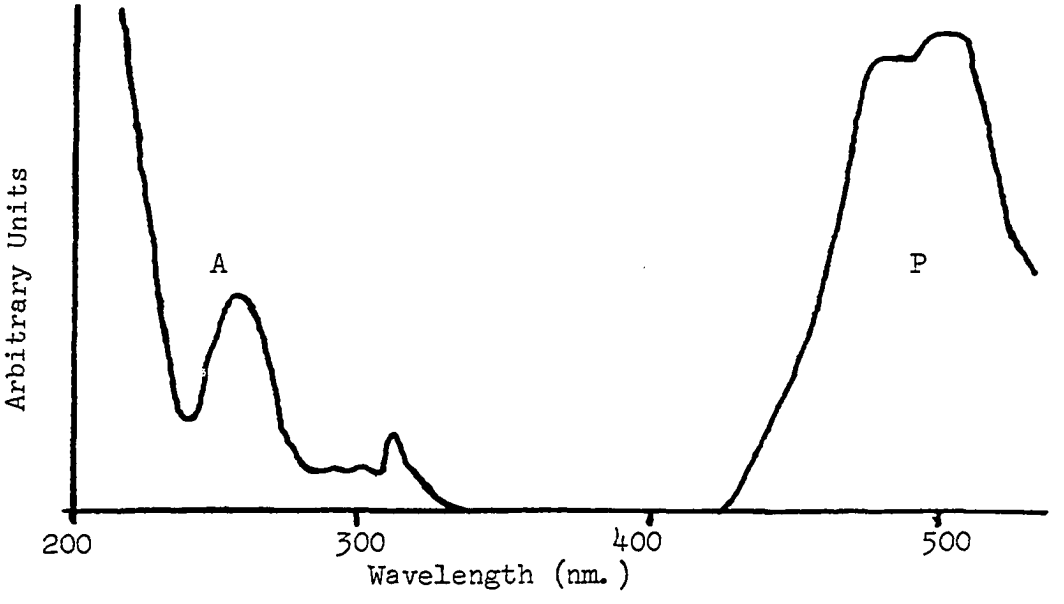

Figure 12. Absorption (A) and phosphorescence (P) spectra of 2-napthalenesulfonamide. P was excited at 250 nm.

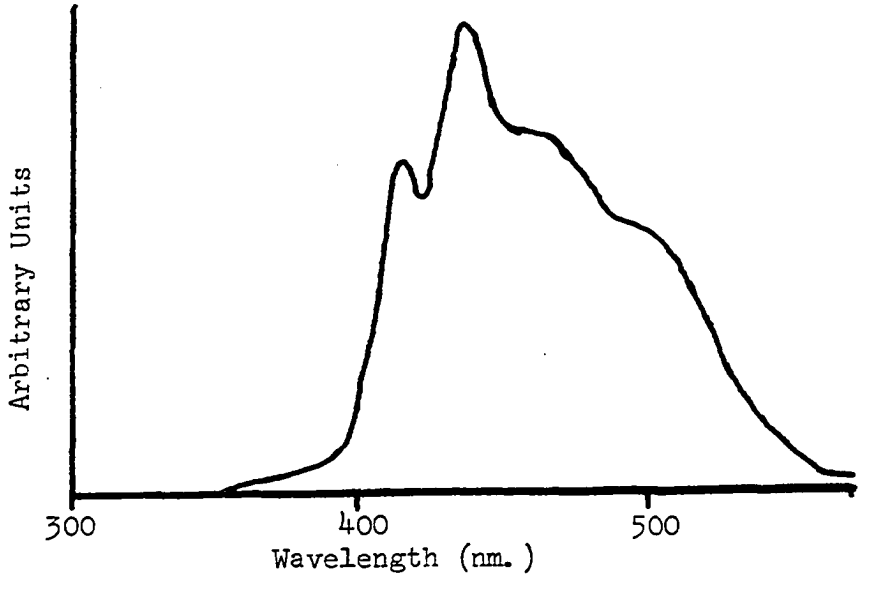

Figure 13. Phosphorescence spectrum of a standard solution of 2-napthalenesulfonamide and carbonic anhydrase excited at 250 nm.

of light absorbed by the enzyme.

Case 3 Inhibitors

The following compounds are all case 3 inhibitors: 4-Carboxybenzenesulfonamide (VI), spectra illustrated in figures 14 and 15; 4-carbamylbenzenesulfonamide (VII), spectra illustrated in figures 16 and 17; 3-carboxybenzenesulfonamide (VIII), spectra illustrated in figures 18 and 19; 4-acetamidobenzenesulfonamide (IX), spectra illustrated in figures 20 and 21; 4-bromobenzenesulfonamide (X), spectra illustrated in figures 22 and 23; and 3,4-dichlorobenzenesulfonamide (XI), spectra illustrated in figures 24 and 25. Since they all give similar results, these inhibitors will be treated as a group.

When standard solutions of these inhibitors and carbonic anhydrase were excited at 296 nm. using a 295 nm. sharp cut-off filter, only a tryptophan phosphorescence spectrum was obtained. Under the same conditions it was found that in the temperature range from 77°K to 160°K only a tryptophan phosphorescence spectrum could be obtained. Therefore, it was not possible to thermally induce transfer from a tryptophan in the active site of the enzyme to these bound inhibitor molecules.

Table 2 summarizes the results obtained by exciting standard solutions of these inhibitors and carbonic anhydrase at 250 nm. It was found that in each case the phosphorescence of the inhibitor was quenched relative to the fraction of light that was absorbed by the inhibitor at 250 nm.

Table 2. Results for Case 3 Inhibitors

Inhibitor	% Absorption (250nm.)	% Quenching	ϕ_P	P/F
VI	25	65	0.18	0.24, 0.25
VII	15	60	0.081	0.23, 0.24
VIII	8	15	0.16	0.20, 0.23
IX	50	15	0.10	0.20, 0.23
X	9	35	0.063	0.16, 0.19
XI	8	40	0.092	0.18, 0.19

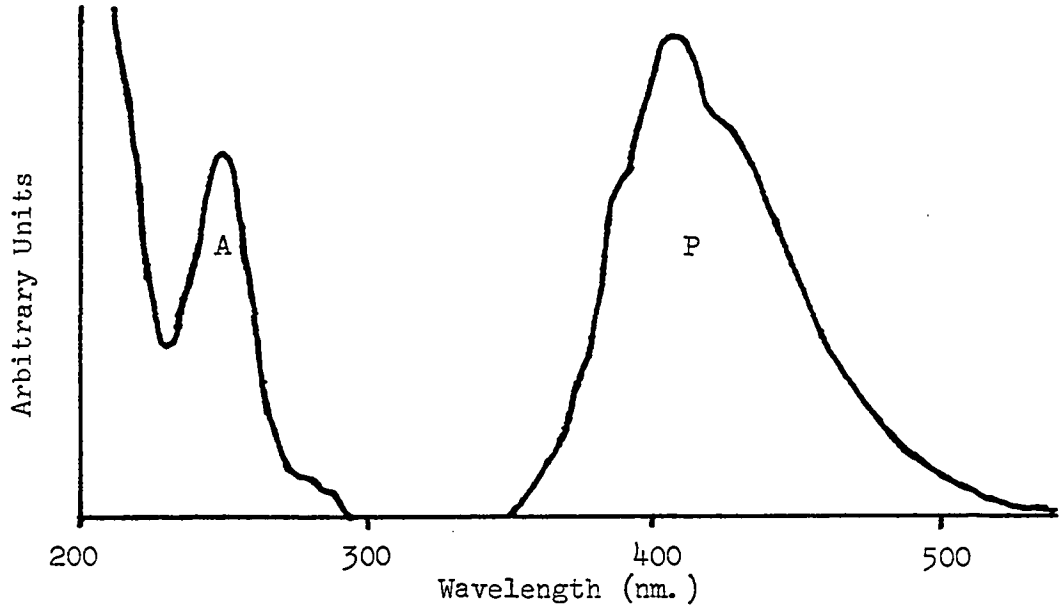

Figure 14. Absorption (A) and phosphorescence (P) spectra of 4-carboxybenzenesulfonamide. P excited at 250 nm.

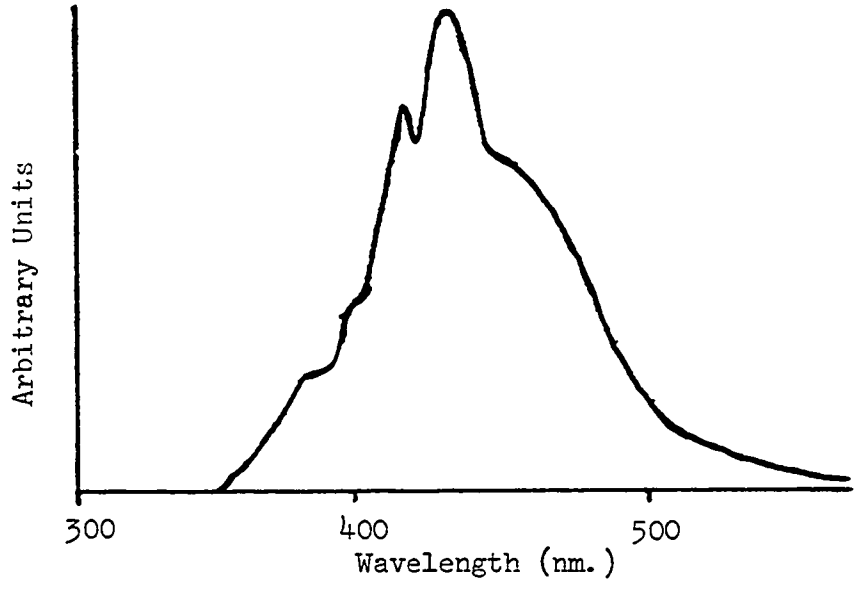

Figure 15. Phosphorescence spectrum of a standard solution of 4-carboxybenzenesulfonamide and carbonic anhydrase excited at 250 nm.

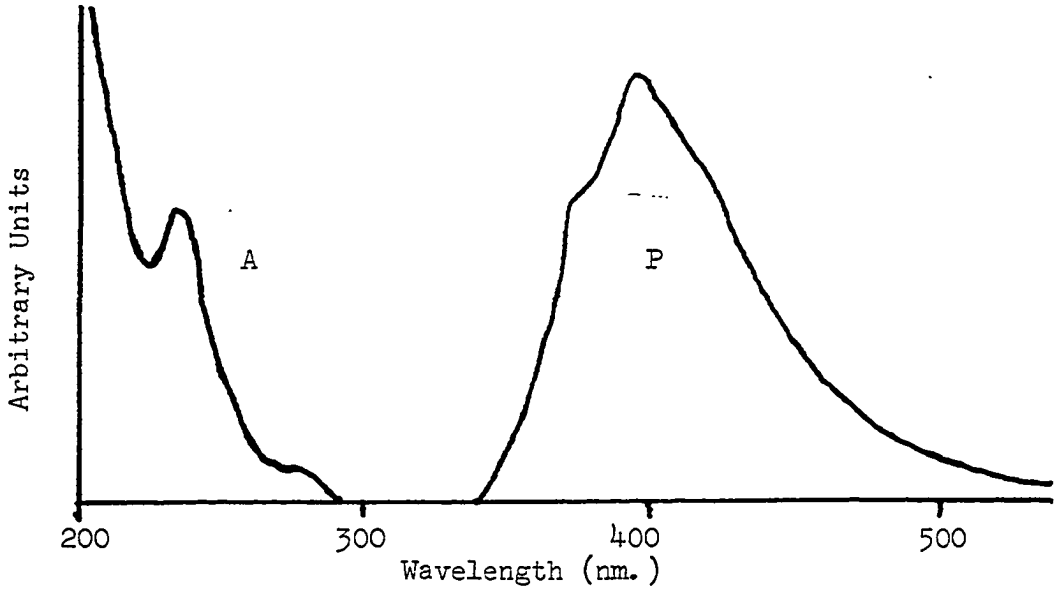

Figure 16. Absorption (A) and phosphorescence (P) spectra of 4-carbamylbenzenesulfonamide. P was excited at 250 nm.

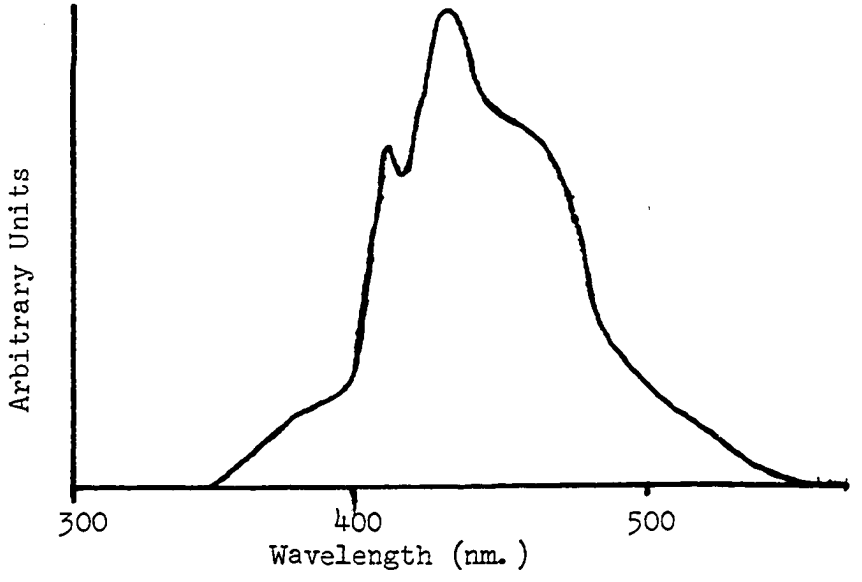

Figure 17. Phosphorescence spectrum of a standard solution of 4-carbamylbenzenesulfonamide and carbonic anhydrase excited at 250 nm.

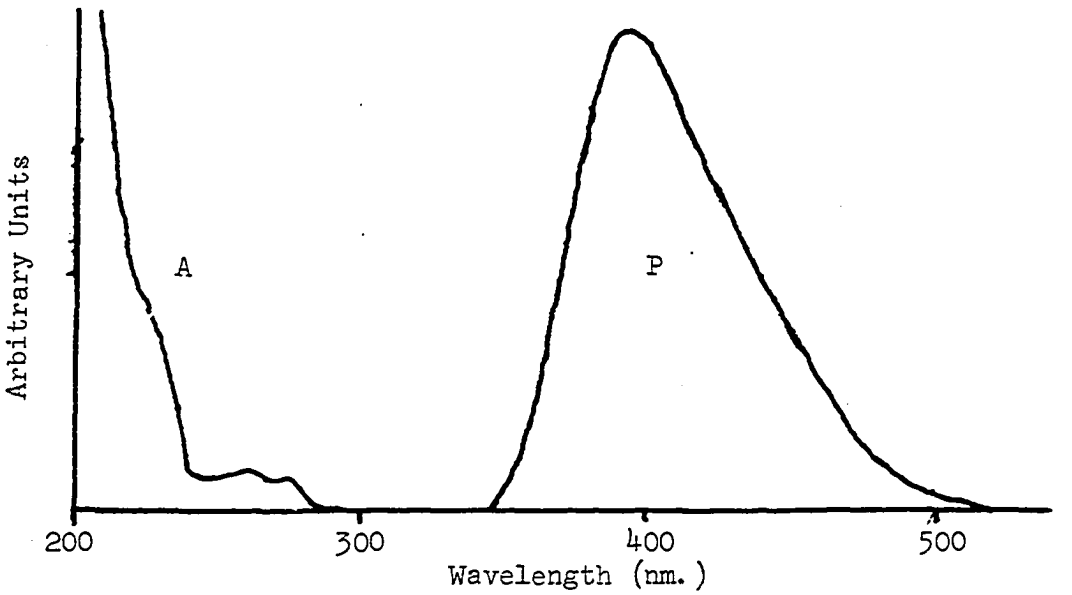

Figure 18. Absorption (A) and phosphorescence (P) spectra of 3-carboxybenzenesulfonamide. P was excited at 250 nm.

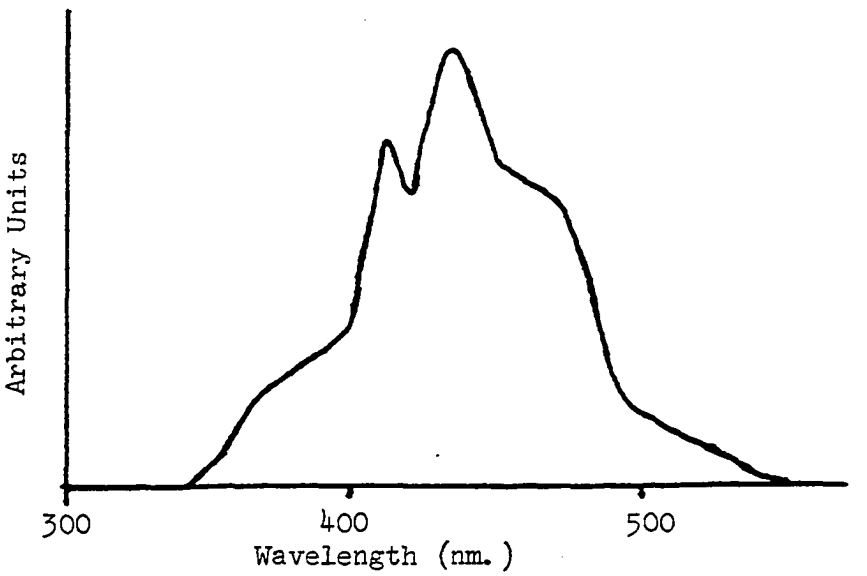

Figure 19. Phosphorescence spectrum of a standard solution of 3-carboxybenzenesulfonamide and carbonic anhydrase excited at 250 nm.

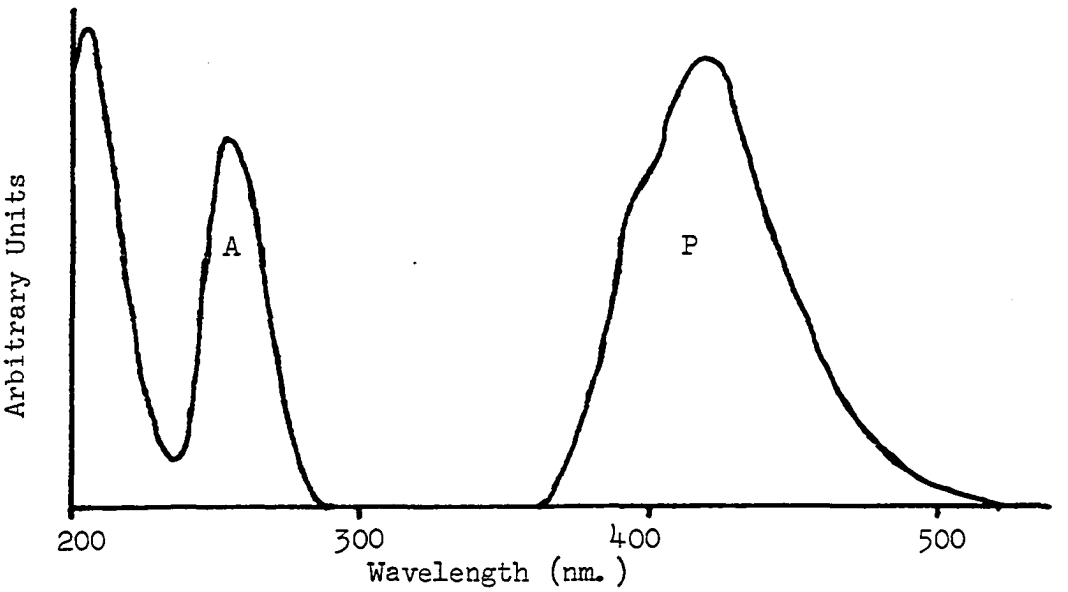

Figure 20. Absorption (A) and phosphorescence (P) spectra of 4-acetamidobenzenesulfonamide. P was excited at 250 nm.

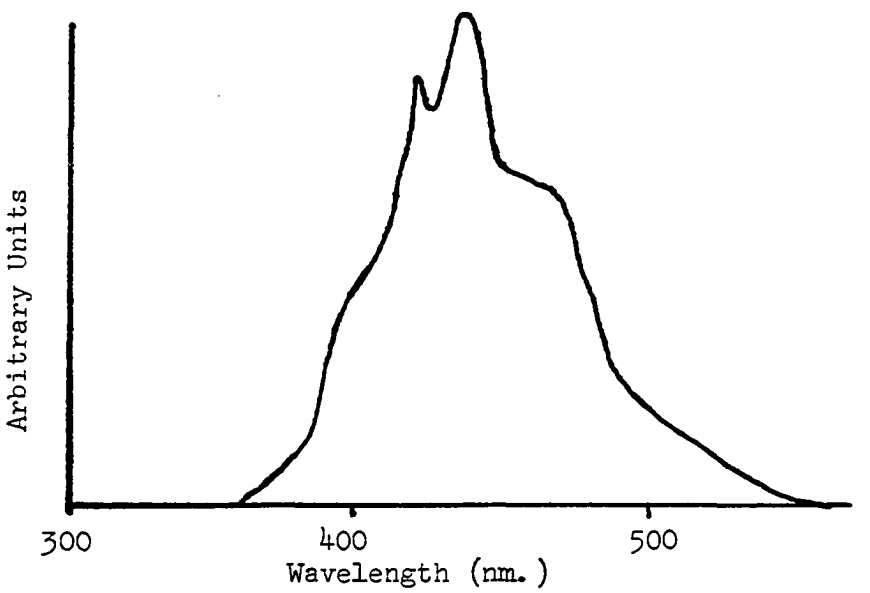

Figure 21. Phosphorescence spectrum of a standard solution of 4-acetamidobenzenesulfonamide and carbonic anhydrase excited at 250 nm.

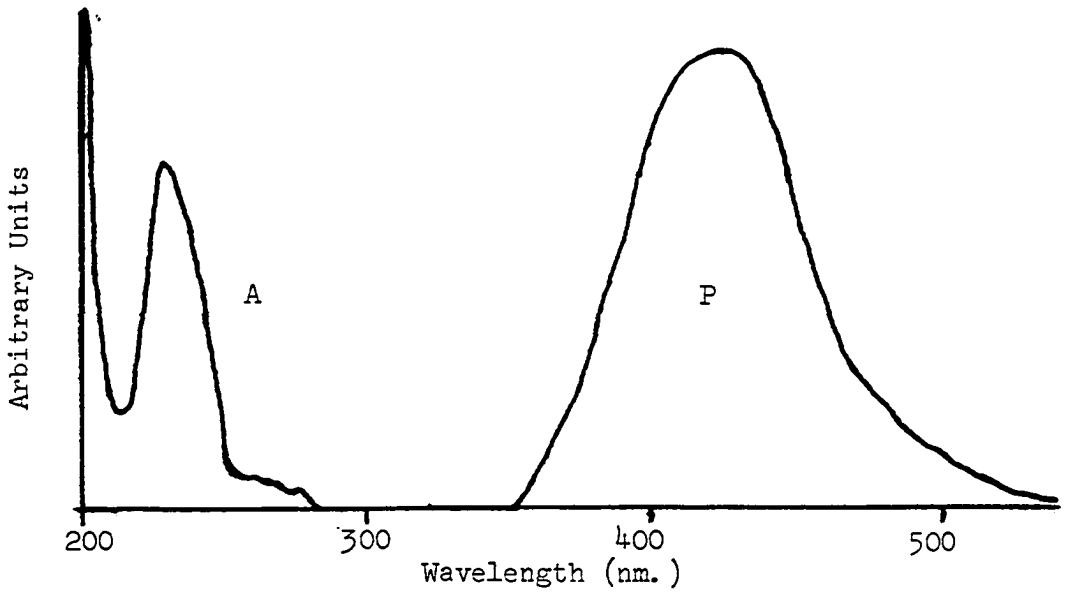

Figure 22. Absorption (A) and phosphorescence (P) spectra of 4-bromobenzenesulfonamide. P was excited at 250 nm.

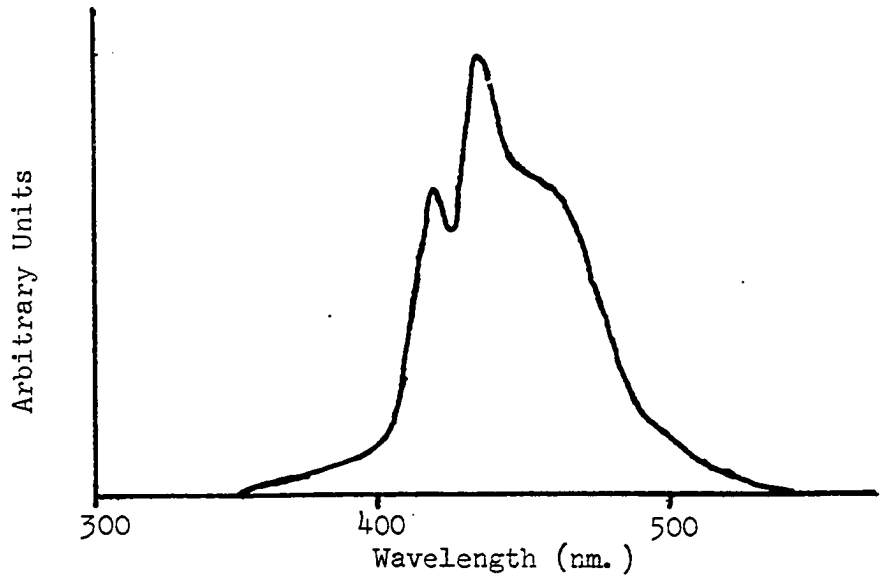

Figure 23. Phosphorescence spectrum of a standard solution of 4-bromobenzesulfonamide and carbonic anhydrase excited at 250 nm.

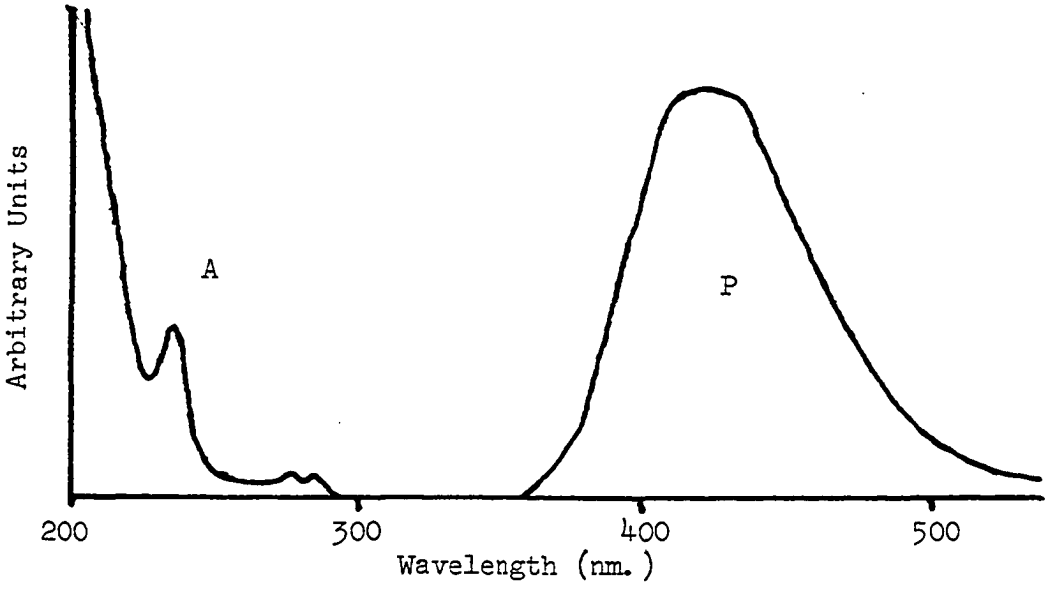

Figure 24. Absorption (A) and phosphorescence (P) spectra of 3,4-dichlorobenzenesulfonamide. P excited at 250 nm.

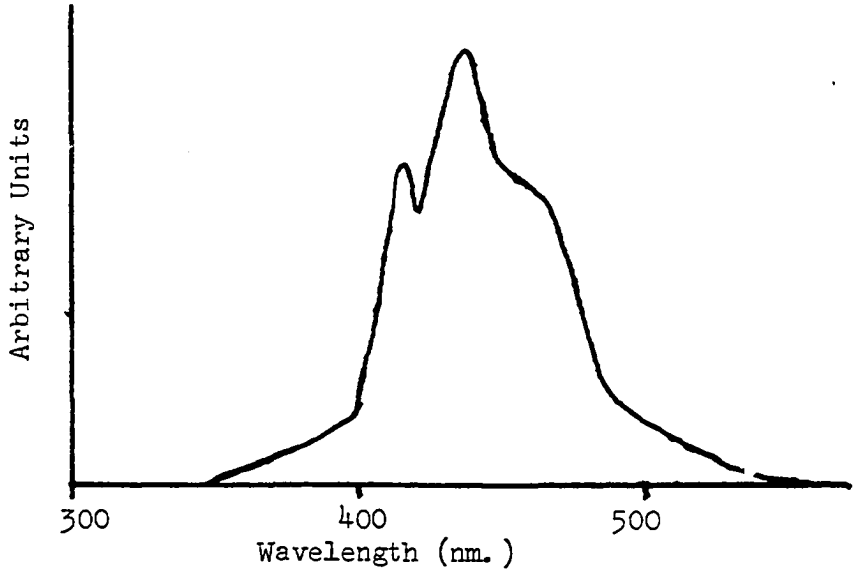

Figure 25. Phosphorescence spectrum of a standard solution of 3,4-dichlorobenzenesulfonamide and carbonic anhydrase excited at 250 nm.

In each case there was an enhancement of tryptophan phosphorescence with no enhancement of tryptophan fluorescence. This gave rise to P/F ratios for tryptophan which were larger than the normal value of 0.15. This demonstrates triplet-triplet energy transfer from each of these bound inhibitors to a tryptophan in the active site of the enzyme.

Benzenesulfonamide, 5-indansulfonamide, 4-chlorobenzenesulfonamide, 4-fluorobenzenesulfonamide, 4-methylbenzenesulfonamide, and 2,4,6-trimethylbenzenesulfonamide are also case 3 inhibitors. In standard solutions of these inhibitors and the enzyme excited at 250 nm., there was no apparent change in the P/F ratio for tryptophan.

DISCUSSION

It can now be said with certainty that there is a tryptophan residue in the active site of one of the isoenzymes of bovine carbonic anhydrase. This statement was previously made by Galley and Stryer in the original work on carbonic anhydrase using inhibitor I. They, however, ignored the possibility of transfer to a tyrosine residue followed by transfer to a more distant tryptophan residue. Since tyrosine to tryptophan transfer is a facile process in proteins, and transfer from I to a tyrosine is energetically favorable, it appears they are not justified in making such a statement.

If the experiments with inhibitor IV are considered, it can definitely be demonstrated that transfer was directly to a tryptophan residue. With IV, transfer to a tyrosine would be an endergonic process while transfer to a tryptophan is an exergonic process. In our later temperature studies we found that even with thermal excitation, endergonic transfer of this type could not be induced. This demonstrates that tryptophan, and not tyrosine, must be located in the active site of the enzyme.

The lack of success in temperature dependence studies with case 2 and 3 compounds was unexpected. Some of these inhibitors had small energy differences between their triplet levels and that of tryptophan, which should have resulted in sufficient thermal excitation to populate the upper triplet state. Apparently no mechanism exists to trap energy at this upper level.

The P/F ratio for tryptophan in standard solutions of the enzyme

was always found to be 0.15, even though the phosphorescent and fluorescent intensities varied by ± 10% due to glass reproducibility. The variation in fluorescent and phosphorescent intensities were always in the same direction, and proportional to each other, so that the P/F ratio remained at 0.15. This is not unexpected as phosphorescence is directly proportional to fluorescence. Any glass effect on fluorescence will have the same proportional effect on phosphorescence giving no apparent change in the P/F ratio of a given chromophore. Hence even a small change in the P/F ratio for tryptophan is significant.

The lack of exact reproducibility in the mixed enzyme-inhibitor systems shown in table 2 is also not unexpected. Since triplet-triplet transfer most probably occurs via overlap of molecular orbitals, it is not unreasonable to expect variation from sample to sample in a mixed system on freezing. The quantity of inhibitor trapped in the proper orientation may be affected by the rate of cooling or small concentration variations. There may also be a small change in enzyme conformation. In any case, it can be seen from table 2 that the reproducibility of P/F ratios in these mixed systems is better than ± 10%.

Referring again to table 2, it is interesting to note that there is a rough correlation between the fraction of light absorbed by the inhibitor, the ϕ_P of the inhibitor, and the change in the P/F ratio for tryptophan. A relatively large inhibitor ϕ_P and per-cent absorption give the largest change in the P/F ratio. A relatively small inhibitor ϕ_P and per-cent absorption give the smallest change

in the P/F ratio. This is exactly what one would expect. The more light an inhibitor absorbs, and the more efficiently this light is used to populate the inhibitor triplet state, the greater an effect there should be on the inhibitors ability to undergo triplet-triplet transfer.

The absence of transfer for some of the case three inhibitors was expected. These inhibitors absorbed only one to four per-cent of the incident light at 250 nm. Therefore, any change in the tryptophan P/F ratio would be undetectable.

It is apparent from this study that triplet-triplet transfer is more facile than singlet-singlet transfer, even though the latter may occur over longer distances. This may be due to an orientation effect, in that the proper orientation for singlet-singlet transfer with the tryptophan cannot be easily attained in the active site of the enzyme.

SUMMARY

Intermolecular triplet-triplet energy transfer between a bound inhibitor molecule and an aromatic amino acid residue in the active site of bovine carbonic anhydrase has been studied. It has been demonstrated conclusively that this aromatic amino acid residue is a tryptophan, and not a tyrosine, by proper choice of inhibitor energy levels.

The inhibitor probe technique has been expanded by utilizing eleven inhibitors having varying energy level arrangements relative to tryptophan. In these studies it has been found that small changes in the P/F ratio for tryptophan are indicative of triplet-triplet transfer. It has also been demonstrated that triplet-triplet transfer is a more common process than singlet-singlet transfer with the chromophores used.

It now remains for this technique to be utilized with other enzymes, and in more detail with carbonic anhydrase when its high resolution x-ray structure becomes available.

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VITA

The author was born on May 19, 1946 in Flint, Michigan. He attended Mt. Morris High School and graduated in 1964. He was married to Margaret Anne Reidy in August of 1968 and was awarded a Bachelor of Arts degree from Western Michigan University in 1969. The same year he began his graduate studies at Western Michigan University and was awarded a teaching assistantship.