A FLUORESCENT PROBE AT THE ACTIVE SITE OF α-CHYMOTRYPSIN[*]

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SUMMARY

A highly fluorescent anthraniloyl group has been inserted at the active site of α-chymotrypsin. The anthraniloyl acyl enzyme is indefinitely stable at neutral pH, allowing a detailed spectrofluorimetric study of the mobility and polarity of the active site. The acylating reagent, p-nitrophenyl anthranilate, is very selective in its reactivity. Only one anthraniloyl chromophore is introduced into chymotrypsin, rendering the enzyme inactive. Chymotrypsinogen, diisopropylphosphoryl chymotrypsin, trypsinogen, lysozyme, and serum albumin do not react at all, while trypsin reacts slowly.

The anthraniloyl chromophore in the acyl enzyme can be selectively excited since its absorption (342 mμ) and emission (422 mμ) maxima are distinct from those of the aromatic residues of the protein. The absorption and emission spectra of methyl anthranilate and other anthraniloyl model compounds vary with solvent polarity. This sensitivity to solvent makes it feasible to determine the polarity of the immediate environment of the acyl group in the enzyme. From the positions of the absorption and emission maxima, as well as from the broadening of those spectra, it is concluded that the environment of the anthraniloyl group at the active site is highly polar. The fluorescence polarization and emission kinetics in the nanosecond range were measured to determine the flexibility of the active site. The rotational

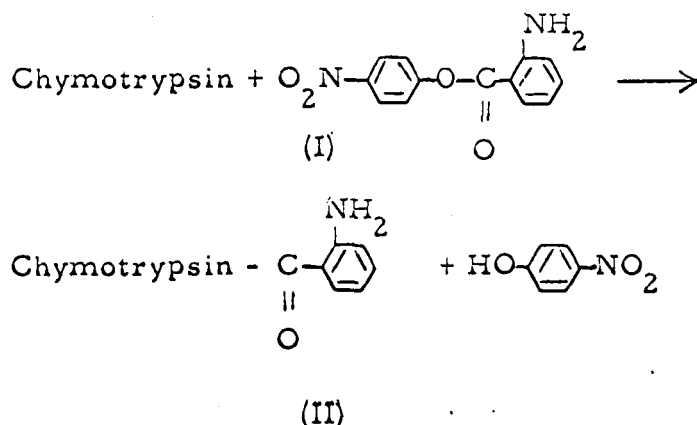relaxation time of the anthraniloyl group is 49 nsec[‡], indicating that
the active site of the acyl enzyme is rigid. .The anthraniloyl chromo-
phore has no rotational mobility independent of the motion of the whole
chymotrypsin molecule. Emission and excitation spectra reveal that
energy is transferred from the tryptophan residues of the acyl enzyme
to the anthraniloyl group with an efficiency of 65%.

1. INTRODUCTION

Fluorescence spectroscopy has proven to be a valuable technique in the study of protein structure and interactions in solution (Velick, 1961; Steiner and Edelhoch, 1962). The method can provide detailed information concerning the active sites of enzymes, if a suitable fluorescent chromophore is located near the active center. The fluorescent group should have an absorption and emission maximum distinct from that of the aromatic residues of the protein, and its excited state lifetime should be sufficiently long so that the rotational mobility of the active site can be determined (Weber, 1953). In addition, it is desirable if the emission properties are sensitive to the polarity of the environment of the chromophore (Stryer, 1965). Finally, the fluorescent probe should be highly selective in terms of its site of attachment to the protein.

We report here a spectrofluorimetric study of the active site of a-chymotrypsin, using a fluorescent probe which meets these criteria. The p-nitrophenyl ester of anthranilic acid (I) reacts specifically with the active site of a-chymotrypsin to form a highly fluorescent anthraniloyl chymotrypsin (II).

Chymotrypsin + O_2N—⟨⟩—O—$\overset{NH_2}{\underset{\underset{O}{\|}}{C}}$⟨⟩ $\longrightarrow$

(I)

Chymotrypsin - $\overset{NH_2}{\underset{\underset{O}{\|}}{C}}$⟨⟩ + HO—⟨⟩—NO_2

(II)

This derivative is stable for months at neutral pH, allowing a detailed study of its spectroscopic properties. Absorption and fluorescence studies of anthraniloyl chymotrypsin have provided a measure of the polarity and rotational mobility of the active site of the active site of the acyl enzyme.

2. EXPERIMENTAL METHODS

(a) Materials :

The synthesis of p-nitrophenyl anthranilate (NPA, I) has been described

by Staiger and Miller (1959). However, we obtained a product which melts

at a significantly higher temperature than previously reported. Commercial

grade isatoic anhyride (29.9 g, 0.183 mole), p-nitrophenol (25.4 g, 0.183

mole) and powdered sodium hydroxide (1.5 g) were heated with 150 ml dioxane

on a steam bath. Carbon dioxide evolution proceeded briskly above 75°.

After 45 minutes, the dark reaction mixture was poured into 75 ml warm

water and cooled, yielding 27.5 g yellow crystals, m.p. $127-128^{o}$ (cf.

Staiger and Miller, m.p. 108°). An additional 10.4 g, m.p. $126-128^{o}$, was

obtained by concentrating the filtrate to near dryness and recrystallizing

the residue from absolute ethanol. The analytical sample recrystallized

from methanol in wide yellow sheaths, m.p. $127.8-128.3^{o}$.

Anal. Calcd for $C_{13}H_{10}N_2O_4$: C, 60.46; H, 3.90; N, 10.85.

Found   C, 60.28; H, 3.80; N, 10.95.

The synthesis and characterization of methyl anthanilate, p-cresyl

anthranilate and anthranilamide will be described elsewhere.

Chymotrypsinogen, α-chymotrypsin (3x recrystallized), diisopropyl-

phosphoryl chymotrypsin, trypsinogen, and trypsin were obtained from

Worthington, while bovine serum albumin and lysozyme were purchased

from Armour.

(b) Anthraniloyl chymotrypsin

Anthraniloyl chymotrypsin (II) was prepared by addition of 1 ml of 4.8×10^{-3} M NPA (4.8 μmoles) in acetonitrile to 20 ml of 2.0×10^{-4} M α-chymotrypsin (4.0 μmoles) in 0.1 M phosphate buffer, pH 6.8, at 5°. The NPA was added in five aliquots at three hour intervals. After an additional 15 hours at 5°, the yellow solution was filtered to remove excess NPA that had precipitated and then dialyzed for two days against several changes of water.

(c) Absorption and fluorescence spectra

Absorption spectra were obtained on a Cary model 14 recording spectrophotometer using 1 cm cell paths. Fluorescence emission, excitation, and polarization measurements were performed on a recording spectrofluorimeter described previously (Stryer, 1965). The emission spectra reported here are the direct recorder tracings which have not been corrected for the variation with wavelength in the sensitivity of the detection system. The relative sensitivity of the 1P28 photomultiplier-grating monochromator system has been experimentally determined to be 3.68, 3.34, 2.97, 2.69, 2.55, 2.29, 1.91, 1.78, 1.59, 1.40, 1.31, 1.20, 1.08, 0.99, 0.89, 0.81, and 0.72 at 10 mμ intervals from 340 mμ to 500 mμ. The corrected fluorescence excitation spectra and polarization measurements were recorded directly using a ratio amplifier. Corning CS 3-73 filters were used to isolate the emission in both the excitation and polari-

zation measurements. Fluorescence spectra were taken at $20.0 \pm 0.2^{\circ}$.

The fluorescence polarization is expressed in terms of the emission anisotropy, A. Jablonski (1960) has emphasized the advantage of using A rather than p, the customary measure of polarization. The emission anisotropy is defined as

$$A = \frac{F_x - F_y}{F_x + 2F_y} \quad , \text{ while } \quad p = \frac{F_x - F_y}{F_x + F_y} \quad .$$

(d) Nanosecond fluorescence kinetics

The excited state lifetime of the anthraniloyl group was measured on a nanosecond fluorimeter designed by Hindley, Garwin, and Stryer. (1966). A flash lamp operated at 12 kV and a repetition rate of about 1 kc provides light pulses which have a width at half-height of less than 3 nsec. The emission was detected by an RCA 1P21 photomultiplier tube operated at 1.3 kV and a Tektronix sampling oscilloscope. Multiple scans (typically 64) of the sampling oscilloscope output are averaged on a LINC computer, which then writes a magnetic tape for detailed analysis on the IBM 7090. Computer analysis of the nanosecond data is necessary because the duration of the light pulse is comparable to the excited state lifetime of many chromophores of biological interest. The observed time course of fluorescence, R (t), is related to the light pulse and instrument response time, L (t'), by the expression

$$R(t) = \int_0^{\infty} L(t') e^{-(t-t')/\tau} \, dt'$$

if the emission is characterized by an exponential decay of lifteime τ. R(t) and L(t') are experimentally observed. τ is then determined by

evaluating $\Sigma L(t')e^{-(t-t')/\tau}$ for various trial values of τ and finding the one

that gives the best least-squares fit to R(t). When the emission is partially

polarized, as in anthraniloyl chymotrypsin, the lifetimes of the parallel

and perpendicularly polarized components differ (Jablonski, 1960). The

mean lifetime depends on the kinetics of the sum of the components, $F_x + F_y + F_z$.

When the exciting light is y-polarized, the total emission is proportional to

$F_y + 2F_x$, since $F_x = F_z$. As noted by Jablonski, (1960), the emission

polarized at 55° to the direction of excitation is in fact proportional to

$(F_y + 2F_x)$. In the present study, a Polaroid type HNB sheet polarizer was

used to obtain y-polarized excitation. The emission was observed at right

angles to the direction of excitation, with a Polaroid HN 32 sheet polarizer

oriented at 55° to y. A Corning CS 7-60 filter was used for excitation,

while the HN 32 polarizer served as the emission filter.

3. RESULTS

(a) Reaction of p-nitrophenyl anthranilate with α-chymotrypsin

Spectrophotometric and spectrofluorimetric measurements reveal that

p-nitrophenyl anthranilate reacts specifically and stoichiometrically with

α-chymotrypsin. When excess p-nitrophenyl anthranilate is added to

α-chymotrypsin, 1.0 equivalent p-nitrophenol is released per equivalent of

enzyme, as determined by following the absorbance of the p-nitrophenolate

ion at 410 mμ. The reaction is complete within thirty minutes and no

additional p-nitrophenol is formed during the next several hours. The

fluorescence emission spectrum (Figure 1) provides further information. Addition of 1.3 equivalents NPA to 10^{-5} M α-chymotrypsin leads to a large decrease in the 333 mμ emission peak and to a large increase in the 422 mμ emission maximum. The half-time of the reaction is about 25 minutes. The 333 mμ peak is due to the fluorescence of the tryptophan residues in α-chymotrypsin, while the 422 mμ emission comes from the anthraniloyl chromophore. The decrease of the 333 mμ peak is due to energy transfer from the tryptophan residues to the anthraniloyl group. The increase in fluorescence at 422 mμ is the result of the release of the p-nitrophenol group, which had completely quenched the fluorescence of the anthraniloyl chromophore in NPA. It is evident that anthranilic acid is not released in the course of the reaction. There is no peak or shoulder at 393 mμ, the emission maximum of anthranilic acid. Furthermore, the existence of an isoemissive point (Anderson and Weber, 1965) at 379 mμ confirms that only two fluorescent species are present.

The constancy of the emission and absorption spectra of the anthraniloyl chymotrypsin following extensive dialysis and gel filtration chromatography indicates that the anthraniloyl group is covalently bonded to the enzyme. Moreover, the complex is stable for months at pH 6.8. The stability of the anthraniloyl chymotrypsin makes it feasible to carry out extensive spectrofluorimetric studies of the modified enzyme.

The enzymatic activity of α-chymotrypsin is virtually completely inhibited by reaction with p-nitrophenyl anthranilate. At pH 6.8, anthraniloyl chymotrypsin has less than 5% of the activity of the native enzyme. The

assay was carried out with glutaryl-L-phenylalanine p-nitroanilide as substrate (Erlanger, Cooper, and Bendich, 1964).

(b) Specificity of p-nitrophenyl anthranilate as a fluorescent labeling reagent

Chymotrypsinogen, diisopropylphosphoryl chymotrypsin, bovine serum albumin, and lysozyme do not react with NPA. Excess NPA was added to 10^{-5} M solutions of these protein. There was no change in absorbance at 410 mμ or in the fluorescence emission spectrum after 24 hours.

Trypsin, but not trypsinogen, does react with NPA, though at a slower rate than chymotrypsin.

(c) Absorption and emission spectra of anthraniloyl chymotrypsin

The absorption spectrum of anthraniloyl chymotrypsin is given in Figure 2. The extinction coefficient is 5.0×10^4 cm^2/mmole at 280 mμ and 4.0×10^3 cm^2/mmole at 342 mμ. The concentration of the solution was determined by amino acid analysis. The observed extinction coefficient at 342 mμ is consistent with a stoichiometry of one anthraniloyl group per chymotrypsin, since the extinction coefficient of methyl anthranilate in a variety of solvents ranges from 3.8×10^3 to 5.4×10^3 cm^2/mmole.

The emission spectrum (Figure 3) shows a maximum at 422 mμ. The absolute quantum yield of the anthraniloyl chromophore when attached to chymotrypsin is 0.53. The emission was excited at 336 mμ, where all of the absorption is due to the anthraniloyl group.

(d) Absorption and emission spectra of model compounds

Absorption and emission spectra of methyl anthranilate, p-cresyl anthranilate, and anthranilamide were obtained in solvents of varying polarity. The solvents were cyclohexane, dioxane, acetonitrile, methanol, and water. The absorption and emission maxima are given in Table I and II.

The emission maxima of these compounds are markedly dependent on the polarity of the solvent. There is a shift of some 40 mμ to the red as the solvent polarity increases from cyclohexane to water. In contrast, the solvent dependence of the absorption maxima is complex. A small shift to the red is observed in going from cyclohexane to dioxane (Table I). However, a significant shift to lower wavelengths is observed in going from dioxane to water. In fact, the absorption maxima in water are at lower wavelengths than in cyclohexane.

The emission spectra of methyl anthranilate in cyclohexane, dioxane, and water are shown in Figure 4. As the solvent polarity increases, there is a broadening of the spectrum as well as a red shift of 2500 cm^{-1}. The width of the emission spectrum at half-height is 3100 cm^{-1}, 3400 cm^{-1} and 3600 cm^{-1} in cyclohexane, dioxane, and water, respectively. A similar broadening of the emission spectrum is observed for the other model compounds. It is of interest to note that for anthraniloyl chymotrypsin (Figure 3), the width at half-height is 3700 cm^{-1}.

The width of the near-ultraviolet absorption band of the model compounds varies with solvent polarity in the same manner. The width at half-height in the absorption spectrum of methyl anthranilate is 3200 cm^{-1}, 3600 cm^{-1}, and 4400 cm^{-1} in cyclohexane, dioxane, and water, respectively. The corresponding width in the absorption spectrum of anthraniloyl chymotrypsin is 4200 cm^{-1}.

(e) Energy transfer

The excitation and emission spectra demonstrate that energy is transferred from the tryptophan residues to the anthraniloyl group. The emission spectrum (Figure 1) shows that the tryptophan fluorescence is quenched, while the excitation spectrum (Figure 5) reveals that the energy is in fact transferred to the anthraniloyl group. These spectra provide independent measures of the efficiency of energy transfer.

The efficiency of transfer, T, is related to the intensity of tryptophan emission in native chymotrypsin, F_o, and in anthraniloyl chymotrypsin, F, by the expression

$$T = 1 - (F/F_o).$$

From the emission spectrum, F/F_o is 0.37, and so the transfer efficiency is 63%.

The transfer efficiency can also be obtained from the excitation spectrum of the anthraniloyl fluorescence, using the relationships

$$E_{280} = \epsilon_{280}^a + T \epsilon_{280}^p$$

$$E_{342} = \epsilon_{342}^a$$

where E_{280} and E_{342} are the magnitudes of the excitation spectrum at 280 and 342 mu, ϵ_{280}^{p} is the extinction coefficient of the protein alone, and ϵ_{280}^{a} and ϵ_{342}^{a} are the extinction coefficients of the anthraniloyl group at 280 and 342 mμ. On re-arranging terms,

$$T = \left[\frac{E_{280}}{E_{342}} - \frac{\epsilon_{280}^{a}}{\epsilon_{342}^{a}} \right] \times \frac{\epsilon_{342}^{a}}{\epsilon_{280}^{p}}$$

E_{280}/E_{342} is 8.47 (from Figure 4), while ϵ_{280}^{a}, ϵ_{342}^{a}, and ϵ_{280}^{p} are 1.0×10^{3}, 4.0×10^{3}, and 49.0×10^{3} cm^{2}/mmole, respectively. These values give a transfer efficiency of 67%.

(f) Fluorescence polarization and excited state lifetime

The fluorescence polarization of anthraniloyl chymotrypsin was measured in order to determine the rotational relaxation time of the active site. On excitation at 360 mμ, the emission anisotropy, A, is 0.211 in 0.1 M phosphate buffer, pH 6.8, at 20°. The enzyme concentration was 3.75×10^{-5} M. A plot of the reciprocal of the emission anisotropy as a function of T/η (Figure 6) gives a straight line which extrapolates to a value of 3.29 at $T/\eta = 0$. This corresponds to a limiting emission anisotropy, A_{o}, of 0.304. T/η was varied by changing the viscosity, η, while the temperature, T, was kept constant at 20°. The viscosity was varied by addition of glycerol to a solution of the enzyme in 0.1 M phosphate buffer.

The rotational relaxation time, ρ , is related to A, A_o, and τ, the excited state lifetime, by the expression (Weber, 1953)

$$\rho = 3\tau \frac{A}{(A_o - A)} .$$

From nanosecond pulse measurements (Figure 7), a value of 7.2 nsec was obtained for τ. These values give a rotational relaxation time of 49.4 nsec.

Sephadex G-75 chromatography of anthraniloyl chymotrypsin was carried out in order to determine whether the modified protein is monomeric or dimeric at the concentration used in these fluorescence polarization measurements. The elution pattern of 3.75×10^{-5} M anthraniloyl chymotrypsin corresponds closely to that of α-chymotrypsin at the same concentration ($V/V_o = 1.6$). The elution volume of ovalbumin, which has a molecular weight nearly twice that of chymotrypsin, was $V/V_o = 1.25$. Thus, anthraniloyl chymotrypsin, like α-chymotrypsin (Safare, Kegeles, and Kwon-Rhee, 1966), is almost entirely monomeric at this concentration.

4. DISCUSSION

A highly fluorescent anthraniloyl group has been inserted at the active site of α-chymotrypsin. The striking experimental finding is that the anthraniloyl acyl enzyme is indefinitely stable at neutral pH, allowing a detailed spectrofluorimetric study of some features of the active site. The acylating reagent, p-nitrophenyl anthranilate, is very selective in its reactivity. Only one anthraniloyl chromophore is introduced into chymotrypsin, rending the enzyme inactive. Chymotrypsinogen, trypsinogen, lysozyme, and serum albumin do not react at all. Enzymatically active chymotrypsin appears to be essential for the reaction, since diisopropylphosphoryl chymotrypsin is not labeled by the reagent. Numerous studies have shown that acylation, carbamylation, and sulfonylation, as well as phosphorylation, all occur at a highly reactive serine residue in the active site (for a review, see Bruice and Benkovic, 1966). It seems likely that the anthraniloyl group also is esterified to this serine residue.

Of the proteins studied, trypsin is the only other one which reacts with p-nitrophenyl anthranilate, though it does so at a considerably slower rate than chymotrypsin. The acylation of trypsin by this reagent is not unexpected, since trypsin and chymotrypsin have overlapping specificities (Inagami and Sturtevant, 1960). It is of interest to know whether other serine proteases react with p-nitrophenyl anthranilate.

The indefinite stability of anthraniloyl chymotrypsin at neutral pH is also a characteristic of sulfonyl, carbamyl, and phosphoryl derivatives of the enzyme. In contrast, most carboxylic acid derivatives of chymotrypsin

deacylate rather rapidly at neutral pH. The most stable acyl enzyme previously reported (Balls, McDonald, and Brecher, 1958) is trimethylacetyl chymotrypsin, which has a half-life of about an hour under these conditions. It remains to be determined whether the stability of anthraniloyl chymotrypsin is typical of anthraniloyl esters per se or whether it is due to a particular interaction of this acyl group with the active site of chymotrypsin.

Hartley and Massey (1956) have sulfonylated the active site of α-chymotrypsin with a fluorescent chromophore. They found that dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) reacts preferentially with a residue at the active site. In comparing dansyl chloride with p-nitrophenyl anthranilate, we find that the latter reagent is more suitable as a fluorescent probe of the active site of chymotrypsin. An advantage of p-nitrophenyl anthranilate is that it reacts only at the active site, while dansyl chloride reacts with other residues as well. This makes it more difficult to obtain a homogeneous derivative in which all of the chymotrypsin molecules contain a fluorescent group at only a single residue.

The fluorescence polarization and nanosecond kinetic studies of anthraniloyl chymotrypsin (Figures 6 and 7) provide a measure of the rotational mobility of the active site. The observed rotational relaxation time, ρ, of 49 nsec can be compared with ρ calculated for a model structure. The rotational relaxation time of an anhydrous,

rigid sphere is given by

$$\rho_o = \frac{3\eta V}{RT} = \frac{3\eta M \bar{v}}{RT}$$

where V is the volume of the sphere, M is its molecular weight, $\bar{v}$ is

its partial specific volume, η is the viscosity of the solution, R is the

gas constant, and T is the absolute temperature. For a sphere of

molecular weight 25,000 and a $\bar{v}$ of 0.73, ρ_o is calculated to be 22 nsec,

in aqueous solution at 20°. The observed rotational relaxation time of

a rigid protein molecule will be greater than ρ_o if the protein is hydrated

or if its shape is not spherical. On the other hand, if the protein is not

rigid, the observed ρ will be less than ρ_o. For anthraniloyl chymo-

trypsin, ρ / ρ_o is 2.2. It can be concluded that <u>the active site of the</u>

<u>acyl enzyme is rigid.</u> The anthraniloyl chromophore has no rotational

mobility independent of the motion of the whole chymotrypsin molecule,

which behaves as a rigid particle. This conclusion is consistent with

previous spectroscopic studies of different acyl chymotrypsins (Massey,

Harrington and Hartley, 1955; Berliner and McConnell, 1966).

The absorption and emission spectra of anthraniloyl derivatives

vary with solvent polarity (Table I and II). This dependence makes it

possible to infer the polarity of the immediate environment of the acyl

group in anthraniloyl chymotrypsin. The interpretation of the emission

spectra (Table II) is straightforward. The emission maximum of

anthraniloyl chymotrypsin is at 422 mμ, while methyl anthranilate

emits at 420 mμ in water and at 380 mμ in cyclohexane (Figure 4). The

other model compounds show the same red shift in the emission maxi-

mum with increasing solvent polarity, an effect generally observed for fluorescent molecules that possess a substantial dipole moment in the excited state (Lippert, 1956). Thus, it can be concluded from the position of the emission maximum that the environment of the anthraniloyl group at the active site is highly polar.

The absorption spectra are more complex, since there appear to be two distinct solvent effects (Table I). The absorption maxima of the model compounds shift to the red in going from cyclohexane to dioxane. However, in going from dioxane to water, there is a significant shift to the blue, so that the absorption maxima in water are actually at lower wavelengths than in cyclohexane. The red shift is most likely a solvent polarity effect. The blue shift may be due to hydrogen bonding between the anthraniloyl chromophore and the solvent, but it must be stressed that this interpretation is by no means unique. The absorption maxima of anthraniloyl chymotrypsin, 342 mμ, is at a longer wavelength than that of any of the model compounds in water. This finding suggests that the environment of the acyl group is highly polar but that water is excluded from it.

The polarity of the environment of the acyl group can also be assessed from the breadth of the absorption and emission spectra of anthraniloyl chymotrypsin. The theoretical basis of the relationship between solvent polarity and spectral broadening has recently been given by Marcus (1965). In polar solvents, the absorption and emission spectra of the anthraniloyl

model compounds are considerably broader than in non-polar solvents.
For example, methyl anthranilate has an absorption bandwidth of
3200 cm^{-1} in cyclohexane and 4400 cm^{-1} in water, while its emission
bandwidth is 3100 cm^{-1} in cyclohexane and 3600 cm^{-1} in water.
Anthraniloyl chymotrypsin has an emission bandwidth of 4200 cm^{-1}
and an absorption bandwidth of 3700 cm^{-1}. These spectral broadening
data provide independent confirmation that the environment of the
anthraniloyl group at the active site is highly polar.

It is of interest to compare our conclusions concerning the polarity
of the active site with those derived by other investigators who have
used different probes. The absorption spectrum of trans-cinnamoyl
chymotrypsin indicates that the environment of that group is more polar
than water (Bender, Schonbaum and Zerner, 1962). The high polarity
was attributed to the carboxylate group of an aspartate residue adjacent
to the reactive serine. In contrast, Kallos and Avatis (1966) reported
that the absorption spectrum of p-nitrobenzenesulfonyl chymotrypsin
indicates that the chromophore is situated in a hydrocarbon-like environ-
ment. A similar conclusion was reached for the binding site of proflavine
(Bernhard, Lee and Tashjian, 1966). The finding of a hydrocarbon-like
environment with some chromophores and a highly polar environment with
others is not surprising since the active site of chymotrypsin consists of
distinct binding loci (Hein and Niemann, 1961), which probably differ
greatly in polarity. Thus, each of the probes samples a particular region
of the active site.

Electronic excitation energy is transferred from the tryptophan residues to the anthraniloyl group in the acyl enzyme, as demonstrated by the quenching of tryptophan fluorescence (Figure 1), as well as by the excitation spectrum (Figure 5). The transfer efficiency is 65%. The spectroscopic conditions for dipole-dipole resonance transfer (Förster, 1947) are fulfilled here. Using Förster's equations, it is possible to calculate a distance R_o at which the efficiency of transfer is 50%, assuming that the chromophores are randomly oriented with respect to each other. For tryptophan to anthraniloyl transfer, R_o is calculated to be 29 Å. In Förster's treatment, the rate constant for energy transfer is proportional to the inverse sixth power of the distance between the energy acceptor and donor, so that the transfer efficiency is expected to be 75% at 23 Å, and 25% at 35 Å. Since there are eight tryptophan residues in a-chymotrypsin, it is not possible to interpret the observed transfer efficiency in terms of a distance between the donor and acceptor groups. Rather, when the coordinates of the tryptophan residues become known from an x-ray crystallographic analysis, it will be of considerable interest to determine whether Förster's theory gives good quantitative agreement with the observed transfer efficiency.

ACKNOWLEDGMENT

FOOTNOTE

‡ Abbreviations used: A, emission anisotropy; NPA, p-nitrophenyl

anthranilate; nsec, nanosecond (10^{-9} sec);

ρ , rotational relaxation time; τ , excited

state lifetime.

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Table I

Absorption Maxima of Model Compounds

SOLVENT

	Water*	Methanol	Acetonitrile	Dioxane	Cyclohexane
Methyl Anthranilate	327 mμ	336 mμ	335 mμ	335 mμ	332 mμ
Anthranilamide	316	328	327	331	330
p-Cresyl Anthranilate	334	343	341	340	337
Anthraniloyl chymo-trypsin	342				

* 0.1 M phosphate buffer, pH 6.8

Table II

Emission Maxima of Model Compounds

	Water*	Methanol	Acetonitrile	Dioxane	Cyclohexane
Methyl Anthranilate	420 mμ	405 mμ	391 mμ	390 mμ	380 mμ
Anthranilamide	418	406	393	390	386
p-Cresyl Anthranilate	428	418	402	401	386
Anthraniloyl Chymo-trypsin	422				

* 0.1 M phosphate buffer, pH 6.8

LEGENDS

Figure 1.   The reaction of p-nitrophenyl anthranilate (1.4×10^{-5} M)

with a a-chymotrypsin (1.0×10^{-5} M) is shown by changes in the emission

spectrum on excitation at 290 mμ. The tryptophan emission at 333 mμ

decreases due to energy transfer to the anthraniloyl group, while the

anthraniloyl fluorescence at 422 mμ increases because of release of

p-nitrophenol.

Figure 2.   Absorption spectrum of anthraniloyl chymotrypsin in

0.1 M phosphate buffer, pH 6.8. An absorbance of 1 corresponds to an

extinction coefficient of 10,000 cm^2/mmole for the (---) spectrum or

1,000 cm^2/mmole for the (——) spectrum.

Figure 3.   The emission spectrum of the anthraniloyl chromophore

in anthraniloyl chymotrypsin. The excitation wavelength was 336 mμ.

Figure 4.   The solvent-dependence of the emission spectrum of

methyl anthranilate. As the solvent polarity increases from cyclohexane

(- - - -) to dioxane (-•-•-•) and water (——), there is a large shift to the

red in the emission spectrum. In addition, the emission spectrum becomes

broader as solvent polarity increases.

Figure 5.   Energy transfer from the tryptophan residues to the

attached anthraniloyl group is shown by this excitation spectrum. The

observed excitation spectrum (——) has a peak of high magnitude at

280 mμ, where nearly all of the absorption is due to the aromatic residues

of the protein. The calculated excitation spectrum corresponding to an

absence of energy transfer (----) is strikingly different at wavelengths

below 300 mμ. The efficiency of energy transfer is 67%.

Figure 6.   Fluorescence polarization of anthraniloyl chymotrypsin

in aqueous glycerol solutions. The reciprocal of the emission on anisotropy,

A^{-1}, is plotted as a function of T/η, to extrapolate to a limiting emission

anisotropy A_o at $T/\eta = 0$. The emission was excited at 360 mμ. The

temperature was 20°.

Figure 7.   Nanosecond fluorescence kinetics of anthraniloyl

chymotrypsin: (a) The intensity of the exciting light pulse (———) and of

the fluorescence of the anthraniloyl group (• • •) are shown as a function

of time; (b) A least-squares fit (---) to the observed fluorescence (• • •)
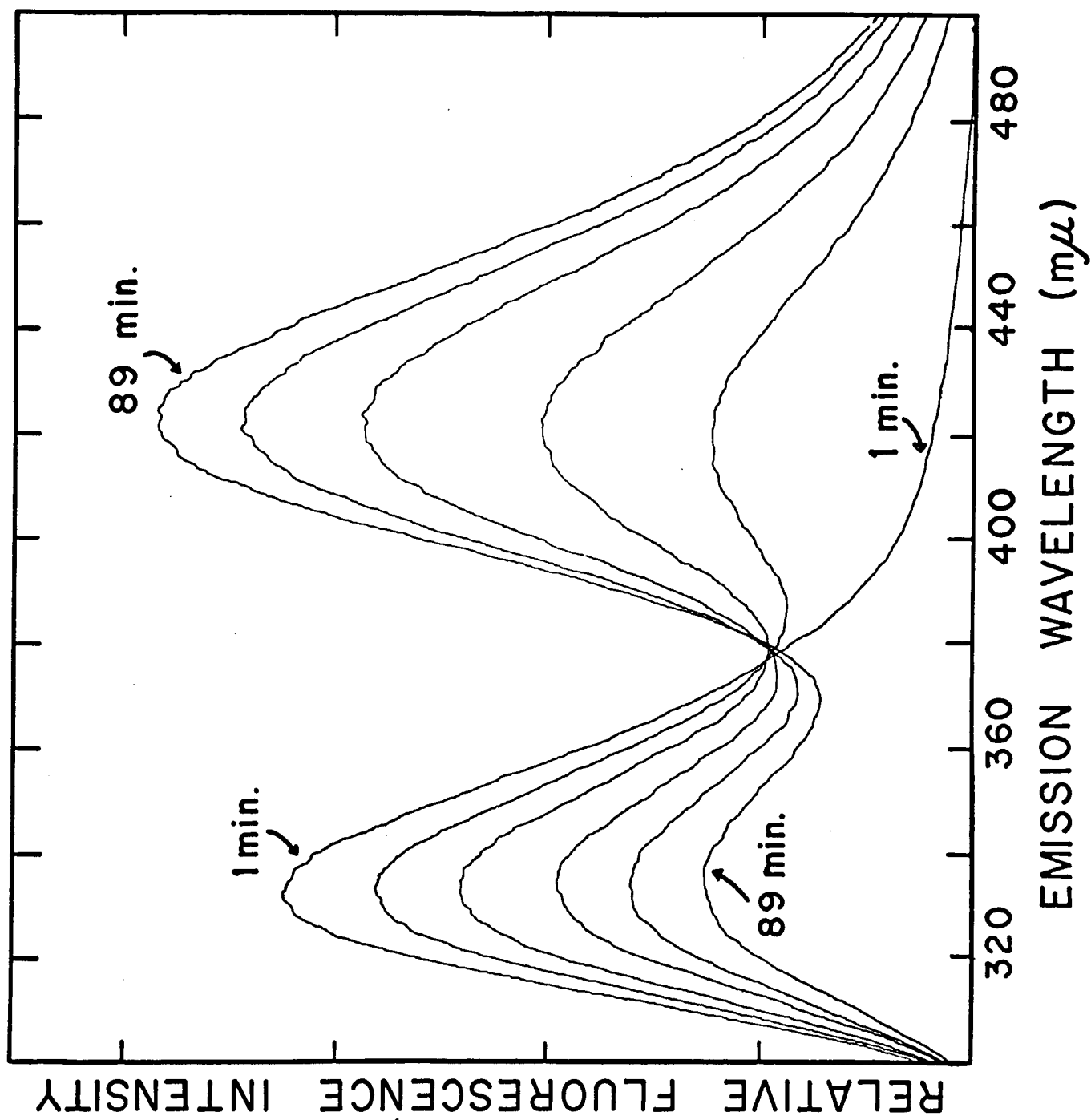
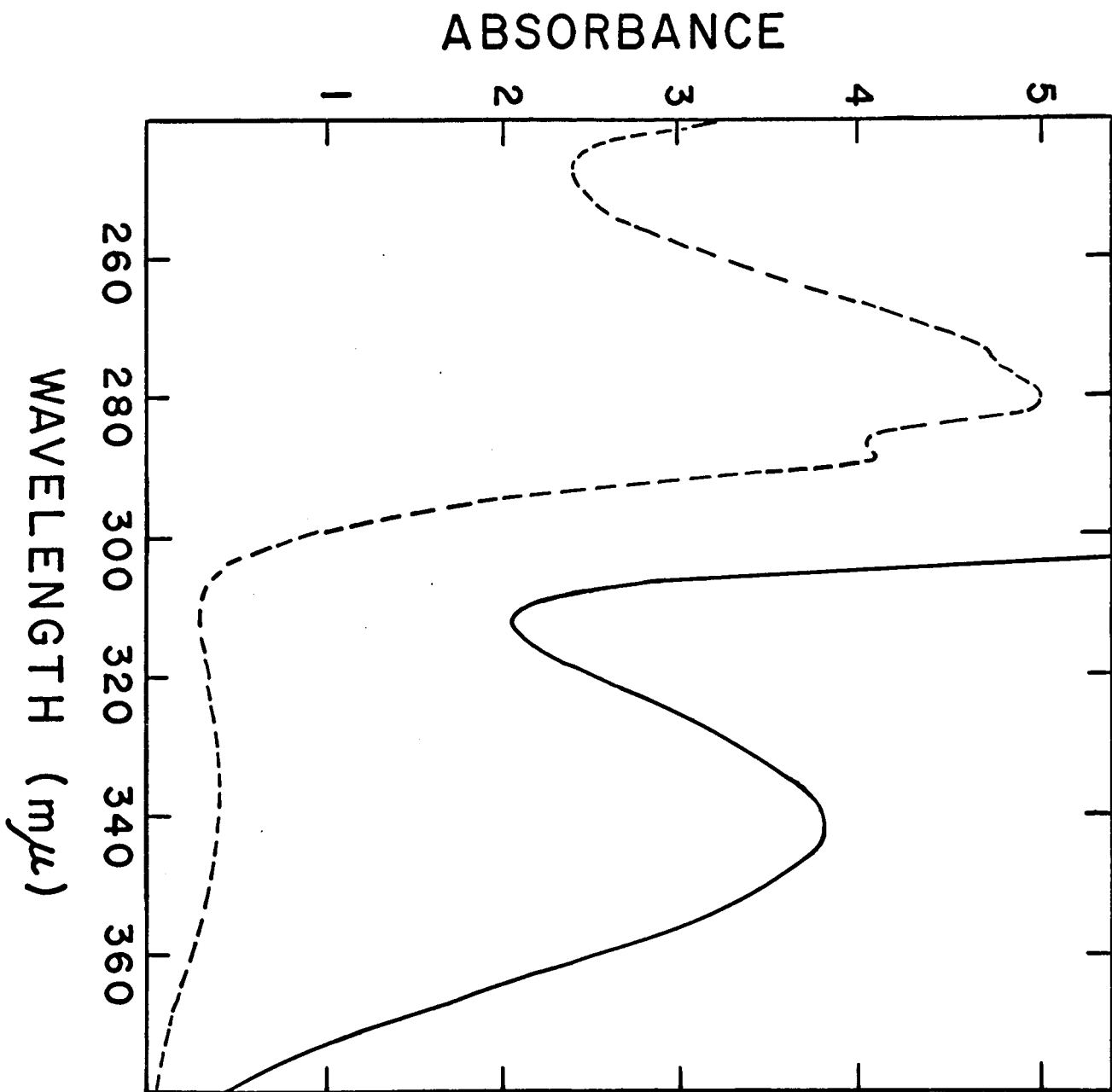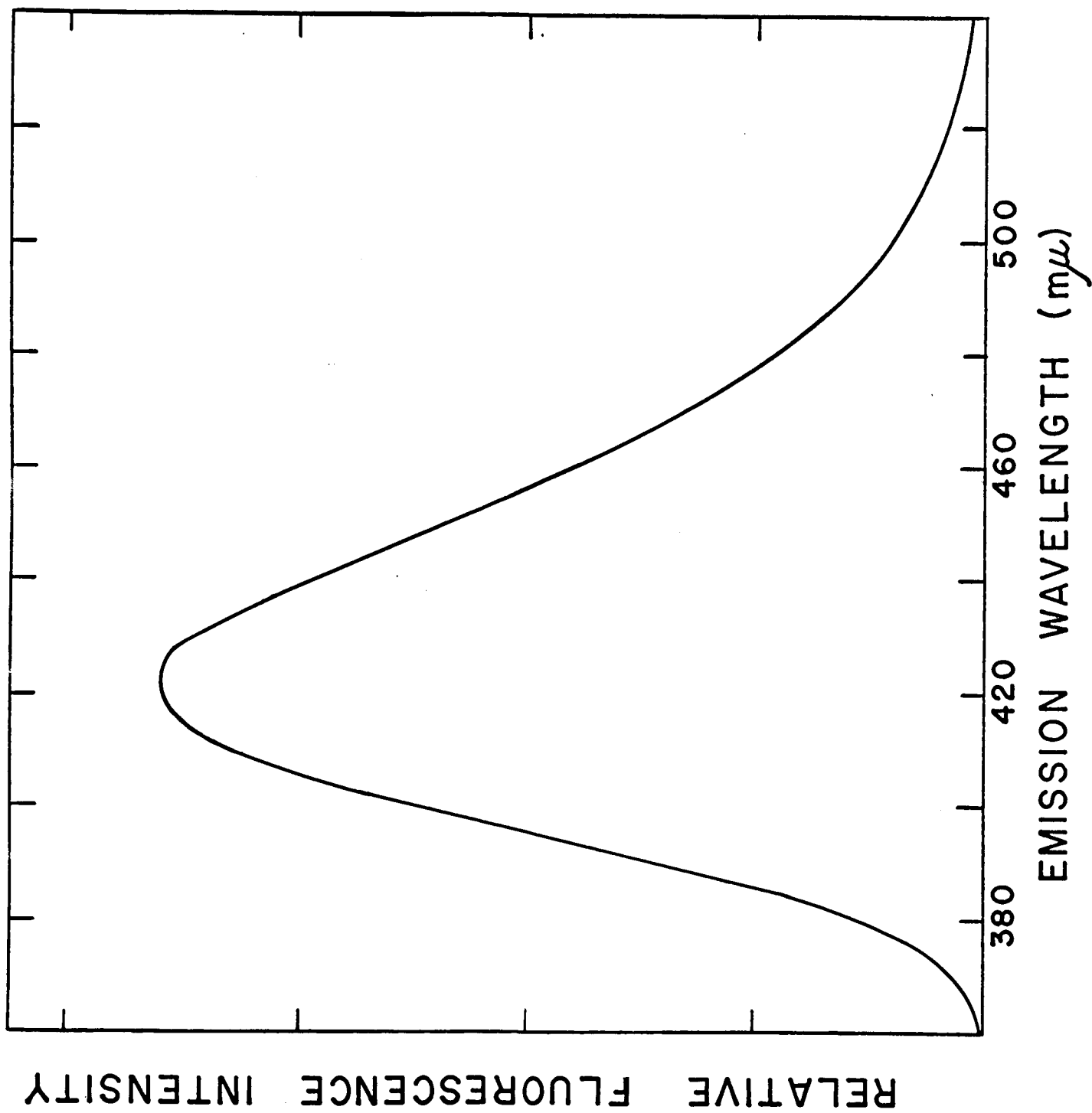yields a lifetime of 7.2 nsec.
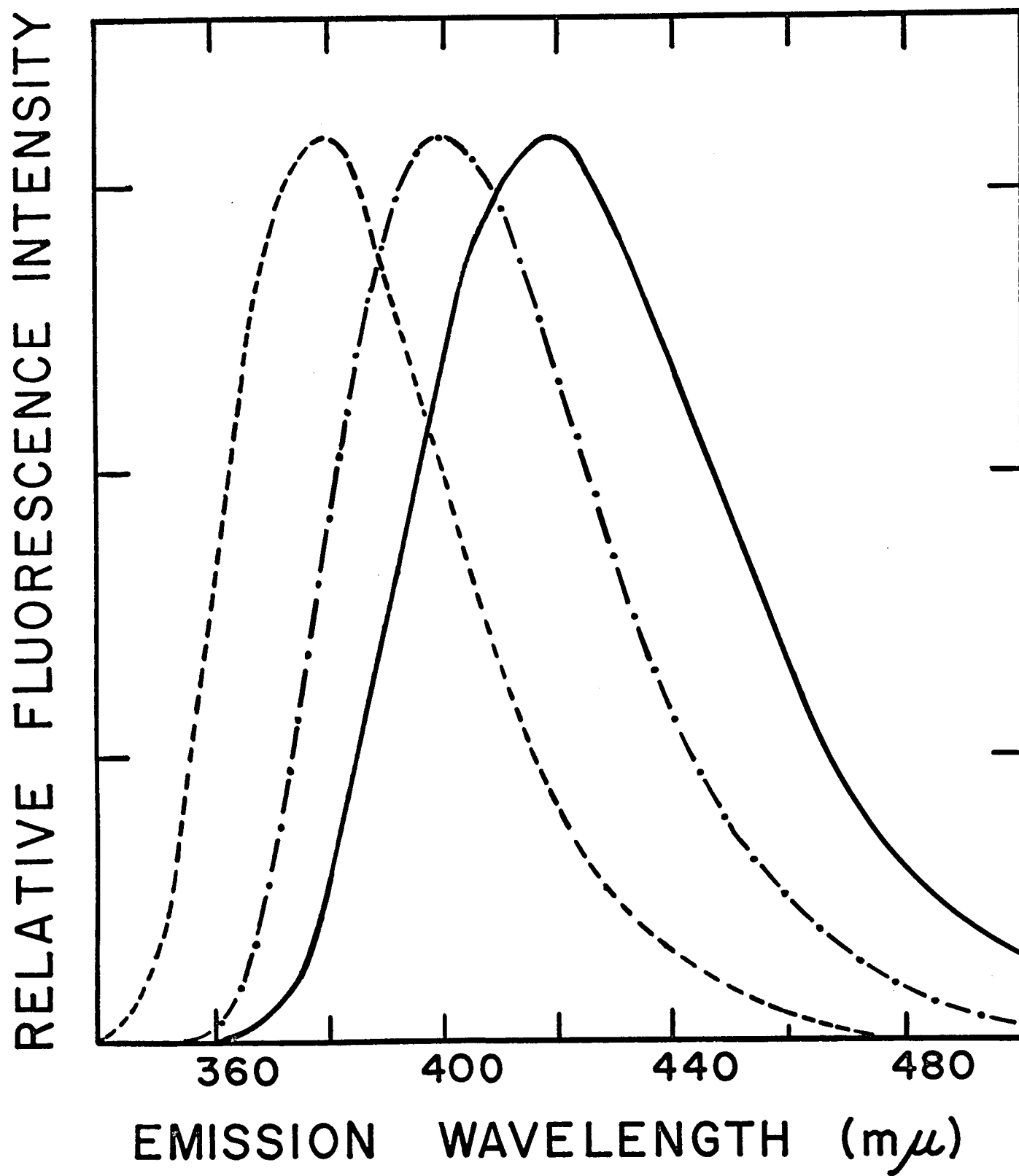
Fig. 1

Figure 2

Fig. 2

Figure 3

Figure 4

Figure 5

RELATIVE FLUORESCENCE INTENSITY

EXCITATION WAVELENGTH (mμ)

280   320   360

Figure 6

Figure 7

LEAST-SQUARES FIT TO AN
EXPONENTIAL EMISSION

τ = 7.2 NSEC

NANOSECONDS

FLUORESCENCE
OF
ACYL ENZYME

EXCITING
LIGHT PULSE

NANOSECONDS

RELATIVE INTENSITY