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The Investigation of the Use of Fluorescein as a Spectral Probe of Protein Environment

Michael Andrew Martin
Union College - Schenectady, NY

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ABSTRACT

The study was iniated to investigate the properties of fluorescein as a reporter group or spectral probe of protein environment.

Through spectral and fluorescence studies it was determined that the fluorescein binding sites were non-polar relative to bulk water. The spectral study also showed a lowering of pK_A for the first one or two fluorescein molecules to bind to the protein. A change in extinction co-efficient showed that there are other differences at the binding site other than the non-polar nature. The fluorescence study showed a quenching of fluorescein fluorescence. This is due to the nature of the binding sites. For latter fluorescein molecules binding to the BSA molecule there was more severe quenching. This could be due to severe quenching at the latter sites or fluorescein to fluorescein energy transfer to a fluorescein bound in a site of very severe quenching.

Due to these changes, one may say that fluorescein would make a good reporter group for protein environment, provided the spectral changes can be correlated to protein environment.

LB

To Zeus

my three month old kitten, who is as dependable at showing up at home as I am at showing up at the Science and Engineering centers.

ACKNOWLEDGEMENTS

I would like to thank Dr. T.C. Werner of the Union College Chemistry Department. It was largely through his seemingly never-ending patience, and his great understanding that this work was completed.

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I would like to thank Dawn Baker for her time at the wee hours of the morning.

Finally, I must extend thanks to the donors of the Petroleum Research Fund (PRF #2399-62), adminstered by the American Chemical Society for support of this research.

Michael A. Martin

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The mind of man is capable of anything - because everything is in it, all the past as well as all the future.

Heart of Darkness, Joseph Conrad

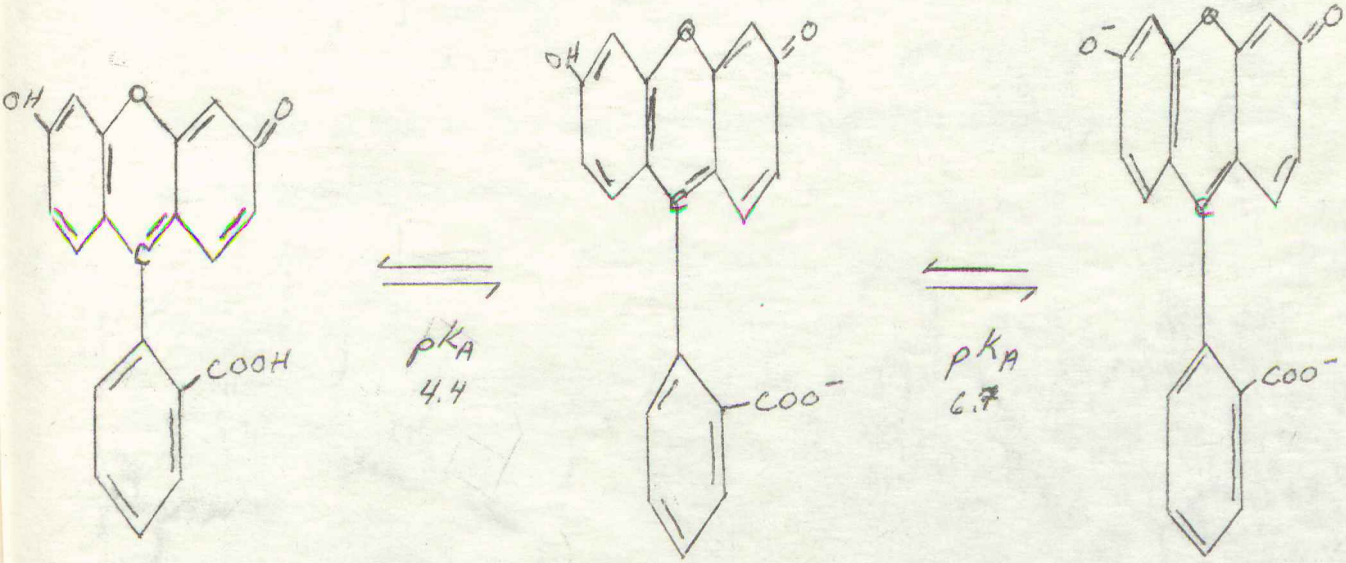
TABLE OF CONTENTS

INDEX OF TABLES AND FIGURES

INTRODUCTION

Werner and Cathou[1] (1971) observed that when fluorescein (Fl) was non-covalently bound to anti-fluorescein anitbodies, the fluorescence of Fl was severly quenched. It was first postulated that this quenching might result from an interaction between Fl and a tryptophan residue in the binding site.[2] However, it was later shown that severe quenching of Fl fluorescence could be observed when the Fl was covalently bound to protein of little or no tryptophan content.[3]

This study was initiated to investigate the properties of Fl as a reporter group or spectral probe of protein environment. Fl has several ionization forms; the most important form for this study is the dianion form (See structures I-III)

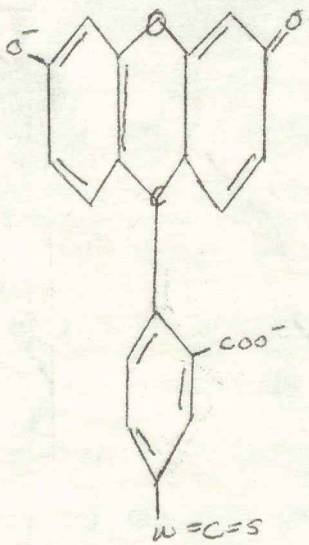


(1) Neutral

(2) Mono-anion

(3) Di-anion

Fl was covalently linked to bovine serum albumin (BSA) and the resulting protein conjugate was used in the study. To link Fl to the protein, fluorescein isotheocyanate (FITC) was used. (see structure IV)



(4)

The -NCS group forms a covalent thioamide linkage with free amino groups on the protein.

EXPERIMENTAL

All common chemicals were obtained from commercial sources and were used without further purification. The BSA and FITC, isomer I, were both obtained from the Sigma Chemical Company, Ionac A-540 was obtained from Matheson Colemen, and Bell. The dialysis tubing was obtained from the Arthur H. Thomas Company.

Ultra-violet and visible absorption measurements were made on a Cary Model 14 spectrophotometer.

Fluorescence measurements were made on a Perkin-Elmer Hitachi MPF-2A spectrofluorometer. Measurements were made at 25°C and in 1 cm cuvets. Solutions were excited at the wavelength of maximum visible absorption. The excitation slit was set at 7 nm and the emission slit at 3nm. The fluorescence spectra were uncorrected for spectral response of the instrument.

The pH measurements were made with a Photovolt Model IIV Electronic pH meter.

The extinction co-efficients were determined for FITC and BSA in 0.1M sodium bicarbonate - sodium carbonate buffer, pH 9.5. The extinction co-efficient of FITC was 8.49×10^4 with maximum absorption at 490 nm. The extinction co-efficient of BSA was 4.25×10^4 with maximum absorption at 280 nm.

The following procedure was used to conjugate the FITC to the BSA. Two milligrams of FITC were added to 100 milligrams of BSA in 0.1M buffer, pH 9.5.

This pH is high enough to neutralize the amine groups on the BSA. This solution was then dialyzed to remove any unbound FITC and the solution was then conjugated two more times in an attempt to increase the FITC to BSA ratio. For the second conjugation, the solution was dialyzed with activated charcoal added to the dialysis buffer to absorb the unbound FITC as it entered the dialysis buffer. This method reduced the dialysis time. For the third conjugation, the solution was passed through a "mini" column containing Ionac A-540 resin to remove excess FITC and then dialyzed. This reduced the time needed for dialysis even more.

The ration of bound Fl molecules per BSA was then determined for the three solutions. It was decided to denature the protein with 0.1N NaOH and then measure the absorption of bound Fl and BSA in 0.1N NaOH. Under these conditions the protein is unfolded and the Fl would have maximum exposure to the solvent. FITC bound to glycine was used as a model of bound Fl. This model has the thioamide linkage and should show any effect of the thioamide bond on the absorption of the bound Fl. The extinction co-efficient of FITC bound to glycine in 0.1N NaOH was found to be 7.74×10^4 with maximum absorption at 495 nm. The extinction co-efficient of BSA in 0.1N NaOH was 6.37×10^4 with maximum absorption at 292 nm. In determining the ration of FITC to BSA, one must subtract out the absorption at 292 nm due to bound Fl from the total absorption at 292 nm. The ration of absorption at 292 of Fl to absorption at 495 of Fl in 0.1N NaOH was 0.28. With

this ratio, one can determine the true absorption of BSA at 292 nm and from this the BSA concentration.

The three conjugated solutions were then dialyzed versus 0.1M acetic acid-sodium acetate buffers, pH 5.0 and 6.0, until they yielded solutions of constant pH. Then a visible spectrum was taken of the three solutions at the different pH's. A solution of Fl was also prepared at the same pH's and visible spectra were taken of these solutions for comparison.

A fluorescence spectrum was taken of the dianion form of each conjugated solution at pH 8.1. A solution of Fl was made at pH 8.1 to be used a standard. The quantum yield of each solution was calculated by using equation I

$$\phi_u = \phi_s \cdot \frac{A_s}{A_u} \cdot \frac{F_u}{F_s} \quad (1)$$

ϕ_u = quantum yield of unknown
ϕ_s = quantum yield of standard
A_s = absorption of standard
A_u = absorption of unknown
F_u = fluorescence of unknown
F_s = fluorescence of standard

As mentioned before the spectra were uncorrected. Since the peaks were similar shape and were used as a measurement of relative fluorescence.

RESULTS

The ratio of Fl molecules per BSA molecule is given in Table I.

Table I
Ratio of Fl Molecules per BSA

Solution	Fl molecules/BSA molecules
Fl-BSA I	1.6
Fl-BSA II	5.0
Fl-BSA III	3.0

The spectra of each conjugated solution and Fl are shown in Figures 1-4. At pH 8.1 the absorption is due to the dianion form. At pH 6.0 there is a mixture of monoanion and dianion forms. At pH 5.0 the monoanion is the predominate form. Note also that the isobestic point present in the Fl spectra (Figure 1) does not appear in the spectra of the conjugated forms.

The absorption co-efficient spectra at the different pH's are presented in Figures 5-7. In Figure 5 a red shift in the spectra of the conjugated solutions relative to the FITC and glycine model spectra is observed. This shift is approximately 2 nm. Note also a decrease in the extinction co-efficient for the Fl-BSA-I solution. There is also a decrease of the extinction co-efficient in for the Fl-BSA II and Fl-BSA III solutions; however it is not as great a decrease

FIGURE 1

Fl solution as a function of pH

(Concentration = 1.23×10^{-5}M
1.0 cm cuvet)

pH 8.1 - (-)

pH 6.0 - (---)

pH 5.0 - (...)

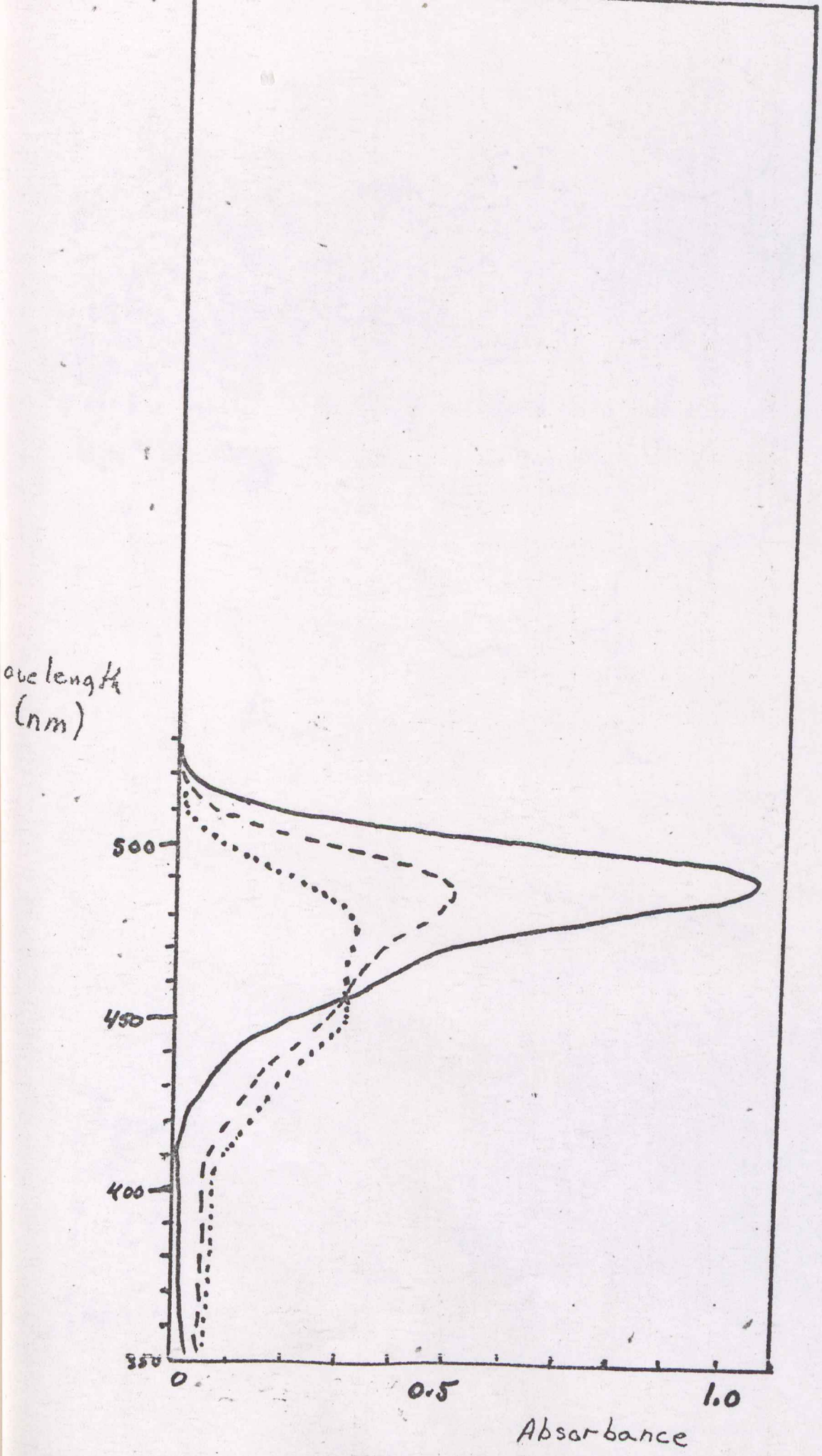

ave length
(nm)
500
450
400
350
0
0.5
1.0
Absorbance

FIGURE 2

Fl-BSA I solution as a function of pH

(Concentration = 1.94×10^{-5}M
1.0 cm cuvet)

pH 8.1 - (-)

pH 6.0 - (---)

pH 5.0 - (...)

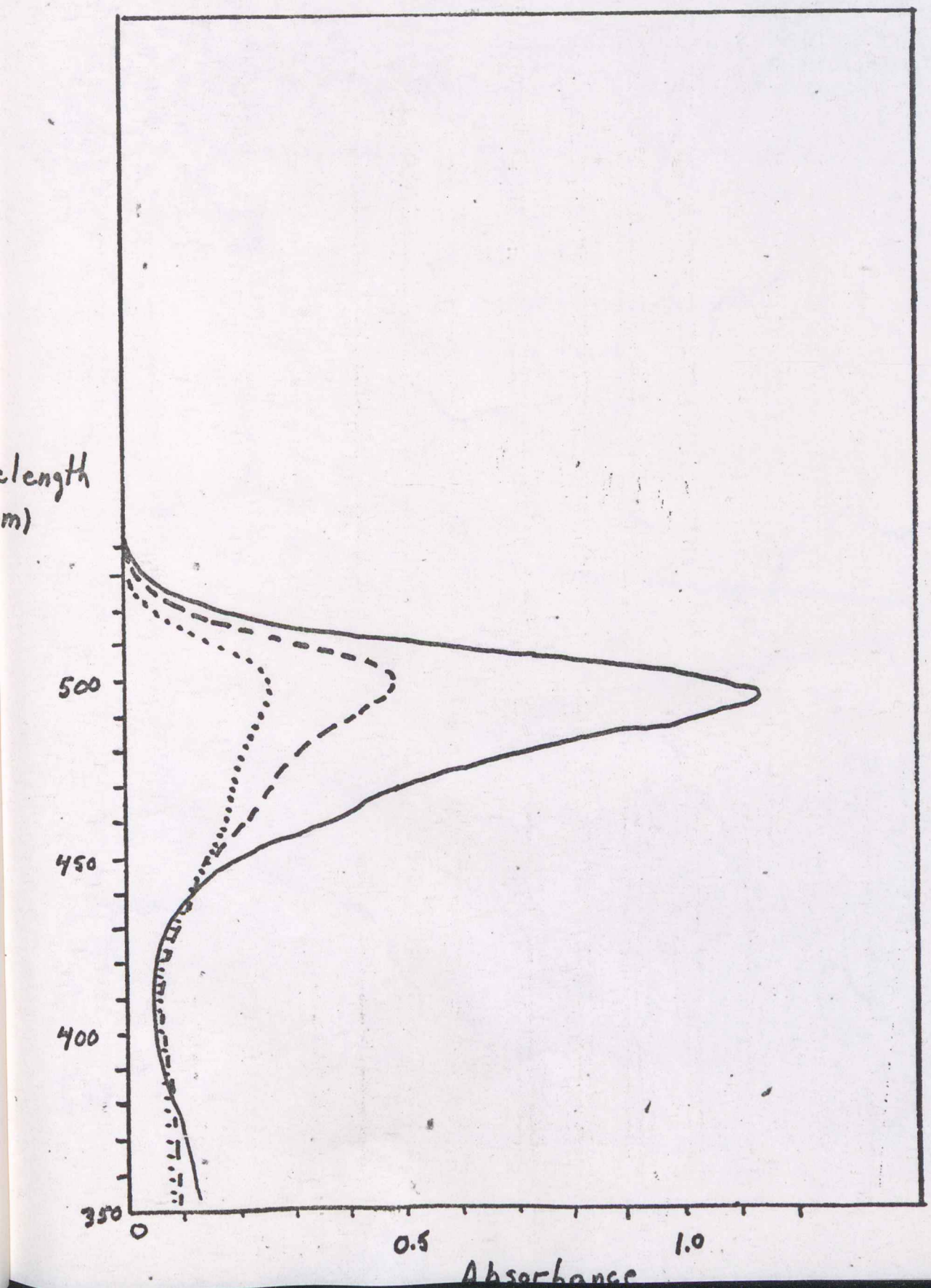

length
m)
500
450
400
350
0
0.5
1.0
Absorbance

FIGURE 3

Fl-BSA II solution as a function of pH

(Concentration = 1.89×10^{-5}M
1.0 cm cuvet)

pH 8.1 - (-)

pH 6.0 - (---)

pH 5.0 - (...)

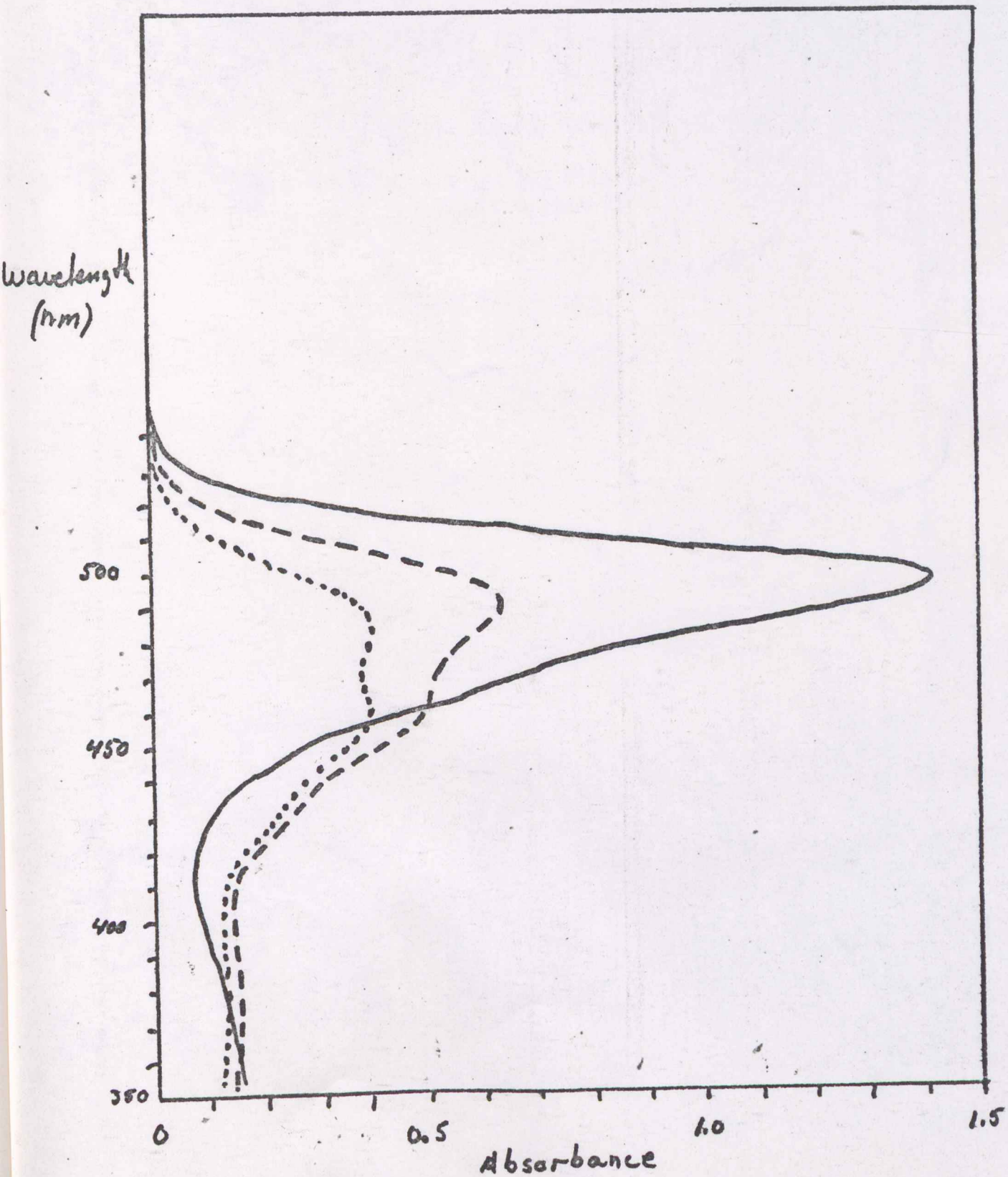

Wavelength (nm)
500
450
400
350
0
0.5
1.0
1.5
Absorbance

FIGURE 4

Fl-BSA III solution as a function of pH

(Concentration = 1.84×10^{-5}M
1.0 cm cuvet)

pH 8.1 - (⊖)

pH 6.0 - (---)

pH 5.0 - (...)

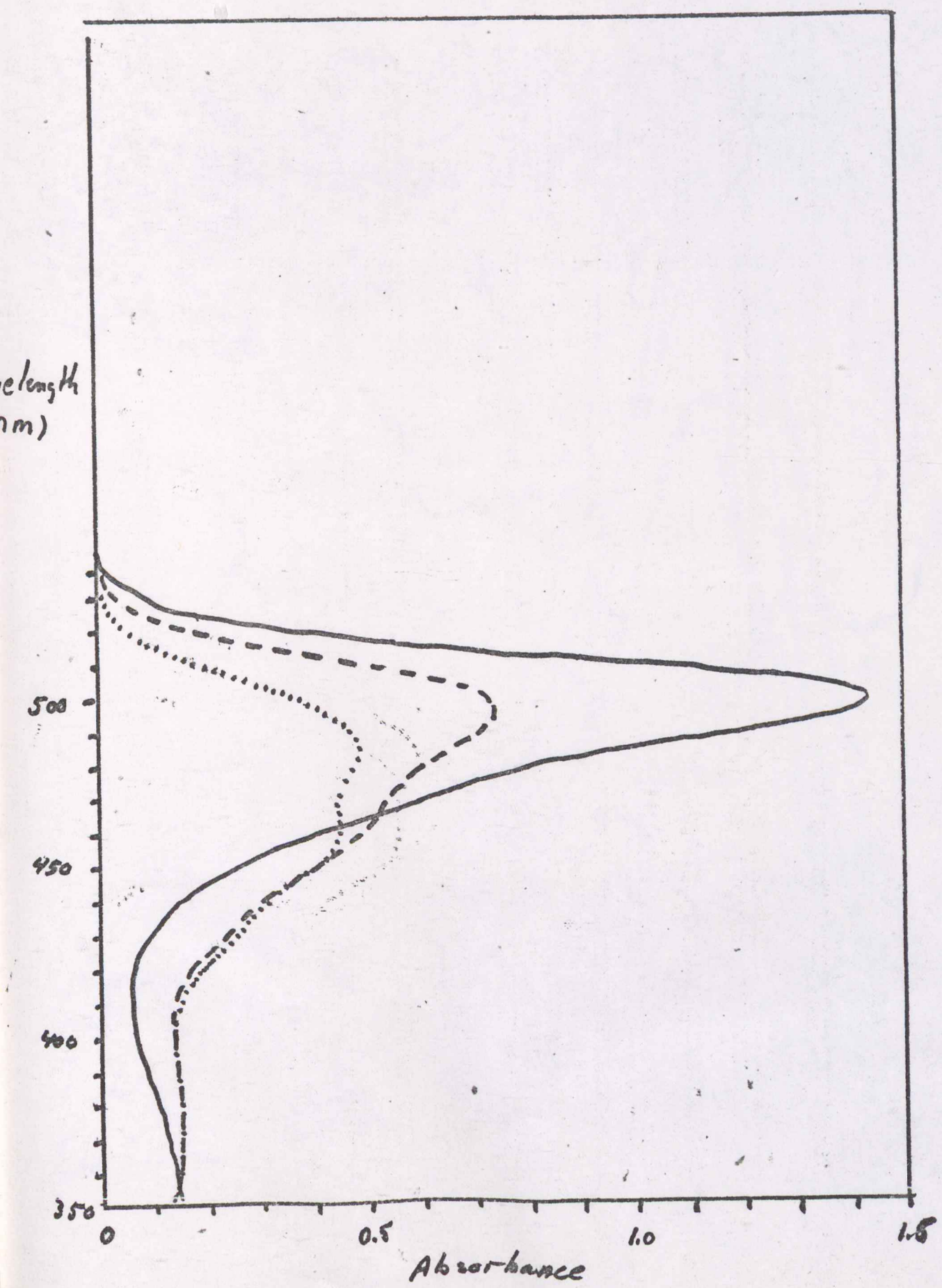

elength
nm)
500
450
400
350
0
0.5
1.0
1.5
Absorbance

FIGURE 5

Absorption co-efficient spectra of Fl and conjugated solutions

(pH=8.1)

Fl	-
Fl-BSA I	0
Fl-BSA II	X
Fl-BSA III	△

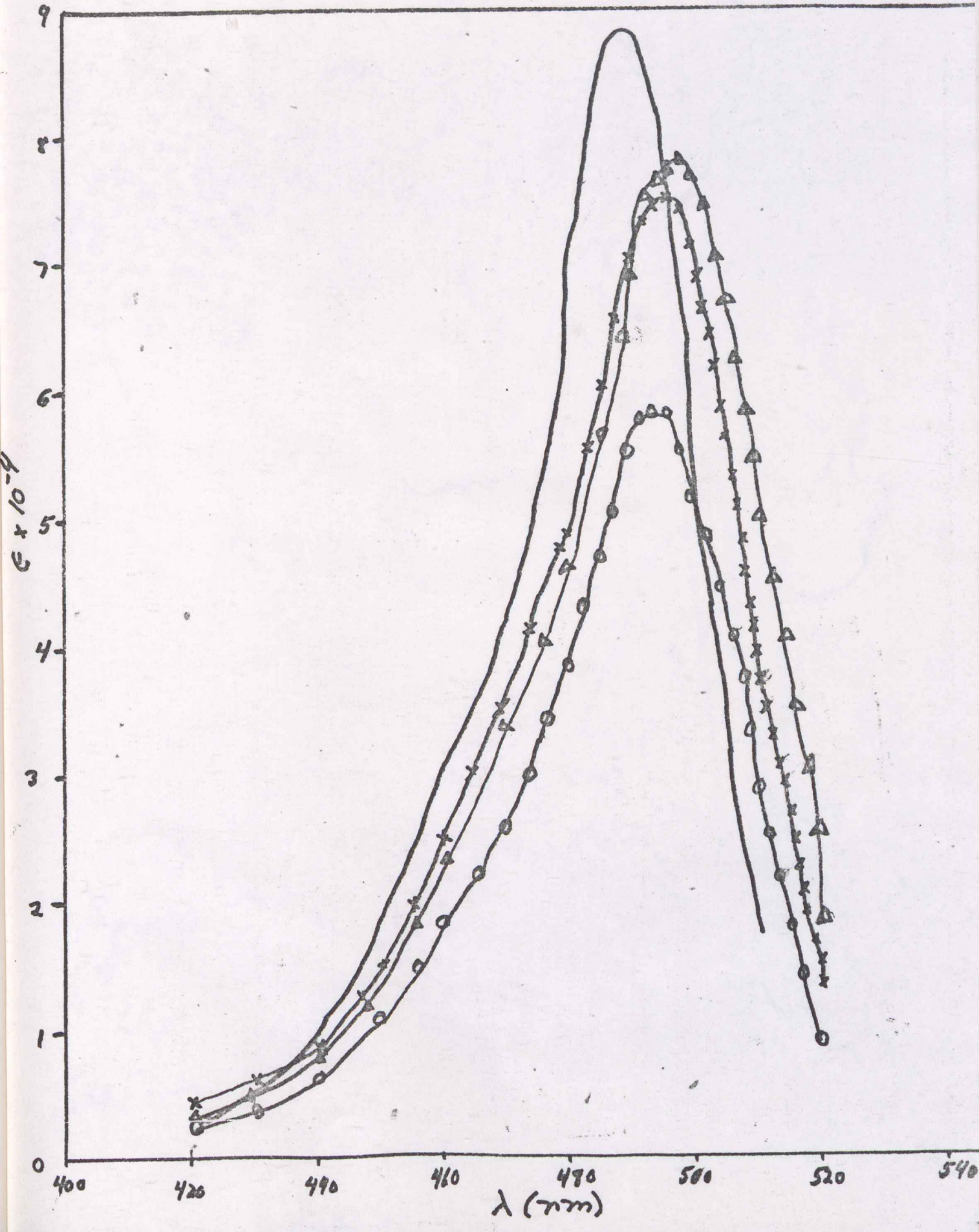

9
8
7
6
5
4
3
2
1
0
ε × 10⁻⁴
400
420
440
460
480
500
520
540
λ (nm)

FIGURE 6

Absorption co-efficient spectra of Fl and conjugated solutions

(pH=6.0)

Fl - —
Fl-BSA I - o
Fl-BSA II - x
Fl-BSA III - Δ

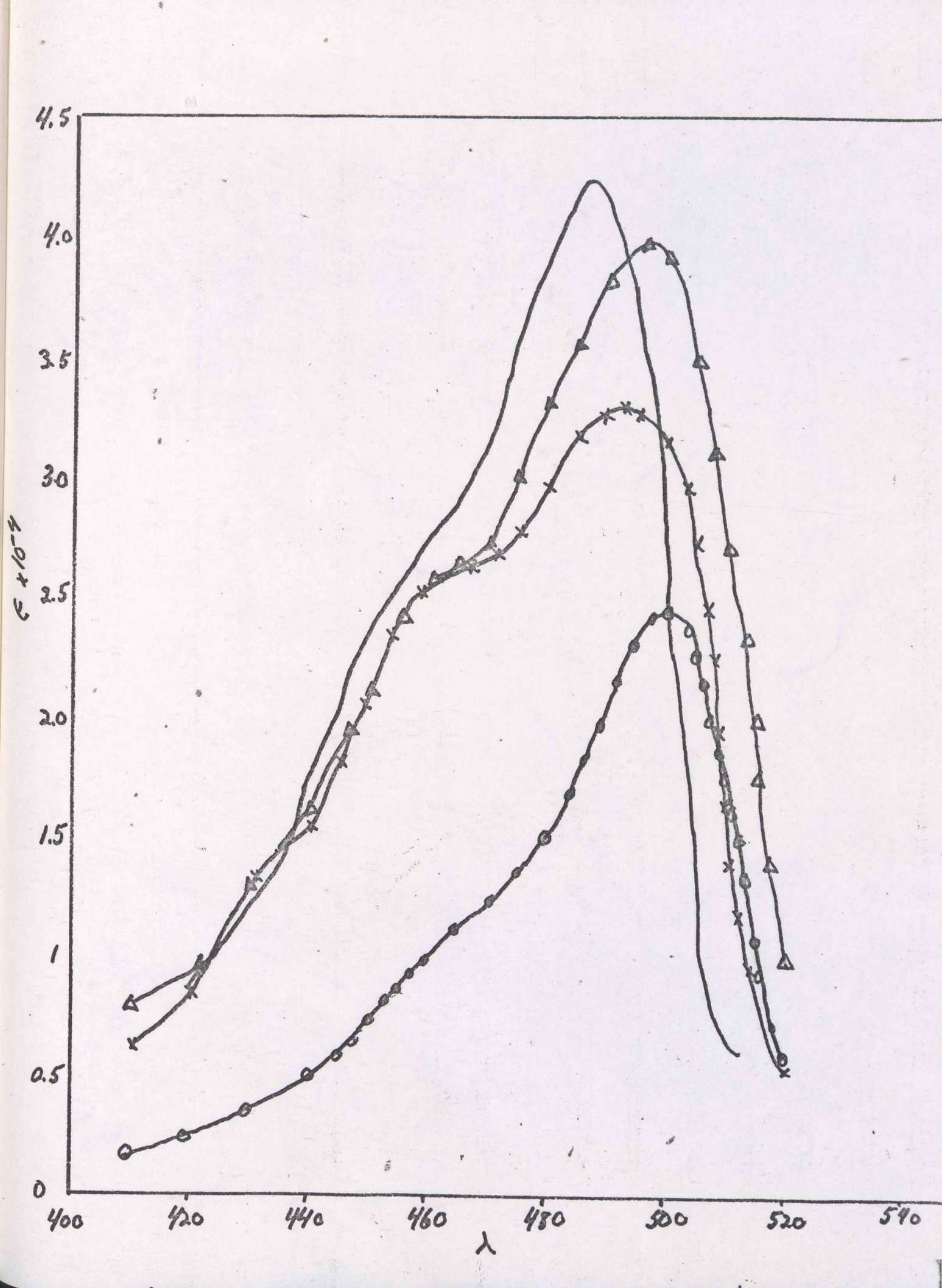

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1
0.5
0
E ×10⁻⁴
400
420
440
460
480
500
520
540
λ

FIGURE 7

Absorption co-efficient spectra of Fl and conjugated solutions

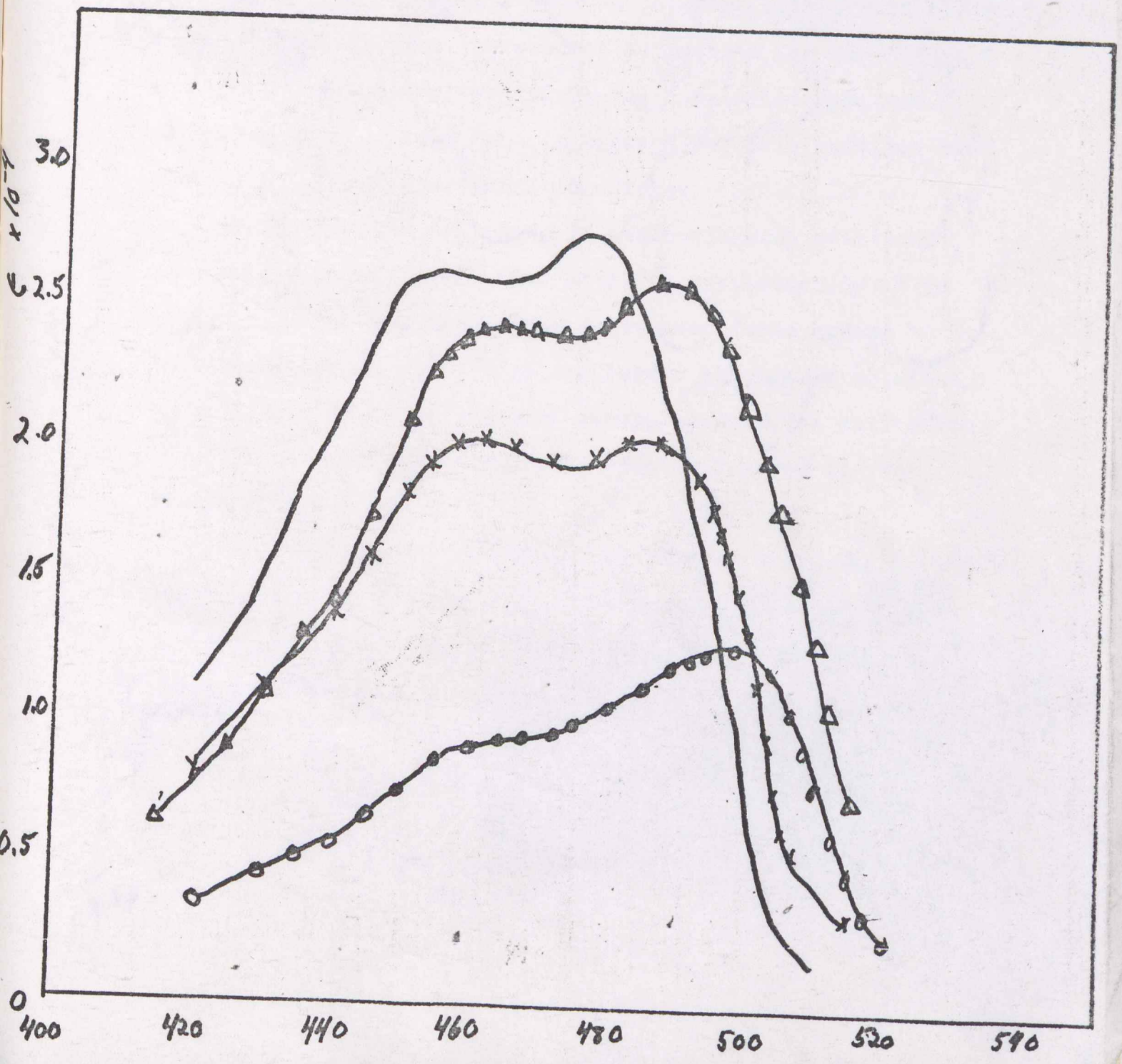
(pH = 5.0)

Fl —

Fl-BSA I – o

Fl-BSA II – x

Fl-BSA III – Δ


3.0
2.5
2.0
1.5
1.0
0.5
0
400
420
440
460
480
500
520
540

as for the Fl-BSA I solution. Others have recorded a decrease of the extinction co-efficient for protein conjugated molecules.[4]

In Figure 6 a red shift of the conjugated solution's spectra relative to Fl spectra is also observed. This shift is between 5 to 12 nm depending on solution relative to Fl. Once again notice the decrease of extinction for the Fl-BSA I solution. Note also that the Fl-BSA I solution spectrum indicates more dianion form is present for this solution than for the other two conjugated solutions.

In Figure 7, a red shift in the conjugated solutions' spectra are again noted. The shift is approximately 10 nm relative to Fl. The two higher conjugated forms appear to be in the monoanion form, while the lowest conjugated solution shows a fair amount of dianion form present. The extinction co-efficient again appears to be greatly lowered for the Fl-BSA I solution.

The fluorescence spectra of the three conjugated solutions and Fl are shown in Figure 8. The quantum yield of Fl was taken to be unity. The quantum yields of the conjugated solutions are presented in Table II.

Table II
Fluorescence quantum yields

$\phi_{Fl} = 1.0$
$\phi_{Fl\text{-}BSA\,I} \sim 0.5$
$\phi_{Fl\text{-}BSA\,III} \sim \phi_{Fl\text{-}BSA\,II} \sim 0.2$

FIGURE 8

Fluorescence spectra of Fl and conjugated solutions

(excitation slit = 7nm
emission slit = 3nm
1.0 cm cuvets, pH = 8.1)

(Fl concentration = 1.23×10^{-7}M)

(Fl-BSA I concentration = 1.94×10^{-7}M)

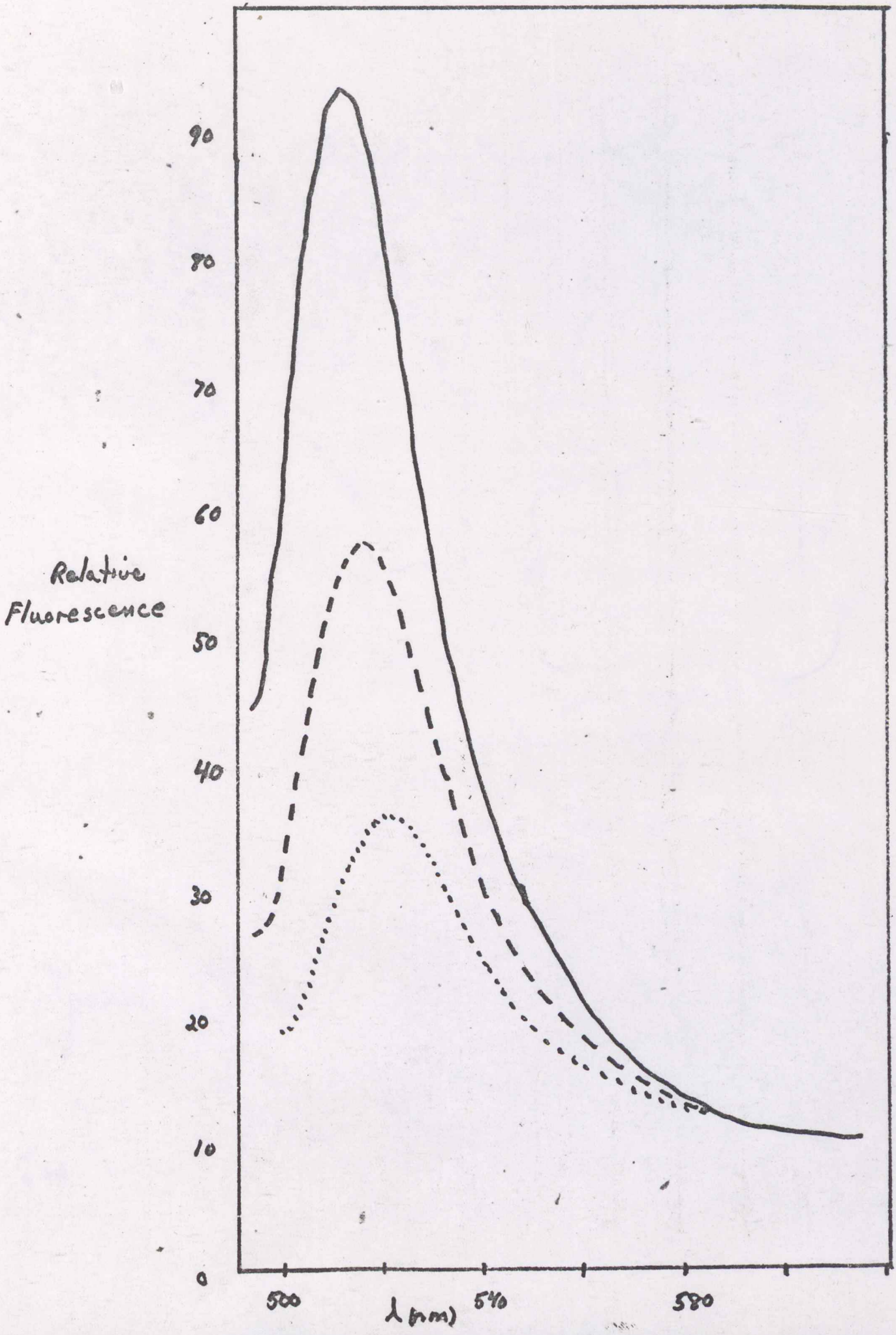
(Fl-BSA II concentration = 1.89×10^{-7}M)

(Fl-BSA III concentration = 1.84×10^{-7}M)

Fl = (-)

Fl-BSAI = (---)

Fl-BSA II = Fl-BSA III - (...)


90
80
70
60
Relative
Fluorescence
50
40
30
20
10
0
500
λ (nm)
540
580

The fluorescence spectra of the conjugated solutions are red shifted relative to Fl by 3-4 nm.

DISCUSSION

From Figures 1-4 one notices a red shift of the conjugated solutions to 497-98 nm at pH 8.1. Free Fl spectra exhibits a red shift in solvents less polar than water.[5] This would indicate that the Fl occupies an environment which is less polar than water. However the red shift is smaller than that observed for antibody bound Fl. Hence the covalently bound Fl is probably more accessible to bulk water than in antibody bound Fl.[6] The lack of an isobestic point in Figures 2-4 may be due to a protein conformational change in the BSA or the fact that the monoanion may be found in a slightly different environment.

In Figures 5,6 and 7 the red shift once again indicates an environment less polar than water. The decrease in extinction co-efficient indicates a different environment for the first conjugated Fl molecules than for latter conjugated Fl's. Since there is little wavelength difference and the extinction co-efficients increase with conjugation, one may assume that the difference in environment between the first conjugated Fl molecule and latter ones is not due to the polarity of the binding site.

In Figures 6 and 7 the lowest conjugated solution appears to have more monoanion present. This would suggest a reduction of the pK_A for the first one or two Fl molecules to bind to BSA. Then the pK_A appears to increase with further conjugation

becoming similar to that of free Fl.

In Figure 8 a red shift of the fluorescence is observed for the spectra of the conjugated solutions. This suggests a hydrophobic environment relative to Fl in water. In Table II a quenching of fluorescence is observed as the conjugatiion increases. The fluorescence quenching for the first Fl molecules bound may be due to the hydrophic environment. It may be also due in part by the lowering of the extinction co-efficient. The extinction co-efficient is proportional to the radiative rate constant (K_f). The lower the radiative rate constant, the smaller the fluorescence quantum yield providing all other rate constants remain the same. There is a severe reduction of Fl fluorescence as more Fl molecules bind to the BSA molecule. This means some of the latter binding sites severly quench Fl fluorescence. Perhaps one of the latter Fl molecules binds in an area of very severe quenching. This molecule may act as a trap for fluorescence from other Fl molecules. Due to the overlap of Fl absorption and fluorescence spectra, energy transfer can occur. The Fl to Fl energy transfer would be effective for separation distances greater than 40Å. A Fl may bind at sites which do not severely quench Fl fluorescence; however, these Fl may be able to transfer energy to the site of severe quenching.

In summary, Fl has spectral properties sensitive to a protein environment. The problem is correlating spectral changes to specific environments. Obviously Fl covalently

bound to BSA does occupy significantly different sites as indicated by the differences in extinction co-efficient, pK_A's, and quantum yields. Due to these changes one could say that Fl would make a good reporter probe for proteins.

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