Fluorescent Properties of Dansylated Bovine Fibrinogen and Correlation of the Heterogeneity of Binding Sites with Heterogeneity and Variability of Fluorescent Properties*

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ABSTRACT: Absorbing and fluorescent properties of dansyl conjugates of bovine fibrinogen of various degrees of labeling were determined. These were compared with the absorbing and fluorescent properties of ε-(1-dimethylaminonaphthalene-5-sulfonyl)lysine and *O*-(1-dimethylaminonaphthalene-5-sulfonyl)tyrosine, the two constituent fluorophores of the labeled protein.

The variations and heterogeneity of the fluorescence of the conjugates can be fully explained on the basis of the fluorescence characteristics of the fluorophores in their free state and their relative proportion in the labeled protein. The environment of the attached label was determined from absorption–perturbation spectra with 60% glycerol and from the quantum yield of the fluorescence. Energy transfer from the chromophores of the native protein to the attached dye molecules was also estimated.

Studies reported in the previous paper (Mihalyi and Albert, 1971) indicated the labeling of both the ε-amino groups of lysine residues and of the phenolic hydroxyl groups of tyrosine residues in dansylated[1] fibrinogen preparations. In the present paper, the absorbing and fluorescent properties of the same preparations will be described. These will be compared with the optical characteristics of the two fluorophores, ε-dansyllysine and *O*-dansyltyrosine, in their free state. This comparison shows that the absorption and the heterogeneity in the fluorescence phenomena of the dansylated fibrinogens can be correlated with the respective properties of the two fluorophores in the free state and their relative proportions in the conjugates.

Fluorescent properties are much more sensitive to environmental factors than the absorption characteristics, therefore, they can be used to advantage in determining the environment in which the label finds itself when attached to the protein (Stryer, 1965; McClure and Edelman, 1966; Turner and Brand, 1968). The fluorescence data obtained were used for this purpose. Also, the energy transfer from the chromophoric residues of the native protein to the attached dye molecules was determined.

Materials

The preparation and some characteristics of the dansyl-fibrinogen conjugates were described in the previous paper. The same preparations were used in the studies reported here and they will be denoted in the same way by dansyl-F-*n*, *n* indicating the number of moles of dye bound per mole of fibrinogen.

The dansylamino acid preparations used in these studies were also described in the previous paper. A highly purified sample of dansylsulfonic acid was obtained from Dr. Chen. Dansylamide was prepared from dansyl chloride by the method of Weber (1952). All other reagents were commercial preparations of Analytical reagent grade.

Methods

Ultraviolet and Visible Absorption Spectra. The Cary Model 14 recording spectrophotometer was used with 1-cm path-length cells. The original solutions served for recording the dye peak at 335 mμ and two successive dilutions were made to have reasonable sizes of the protein and of the low-wavelength dye peaks. The base line of all the spectra was adjusted to zero at 450 mμ where the solutions should have no true absorption. Difference spectra were recorded with the expanded, 0–0.1, slide-wire of the same instrument, with cylindrical tandem cells obtained from Pyrocell Co., Westwood, N. J.

Fluorescence Techniques. Fluorescence excitation and emission spectra were recorded in a Hitachi-Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A, equipped with an R 106 photomultiplier and coupled to a Model QPD 33 recorder of the same manufacturer. Suprasil, 1-cm path-length cells for fluorimetry, from Precision Cells Inc., New York, N. Y., were used in a thermostated cell holder, which maintained the desired temperature to ±0.2°.

Both the excitation and the emission spectra were first corrected for solvent blanks obtained under the same slit and sensitivity conditions. These corrections were low with the usual buffer solutions, but became important in the presence of higher concentrations of glycerol.

Excitation spectra were corrected for variations with wavelength of the light source–monochromator output as described by Melhuisch (1962). A 0.3% solution of rhodamine B in ethylene glycol was used as quantum counter. Correction factors were also obtained by comparing absorption and excitation spectra of dansylsulfonic acid and dansylamide, following the recommendations of Argauer and White (1964). These two compounds can be used in the range from 230 to 350 mμ. Their absorption bands are about 17 mμ apart; nevertheless they yielded identical results. Also, these were

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[1] The 1-dimethylaminonaphthalene-5-sulfonyl group will be abbreviated by the acronym dansyl.

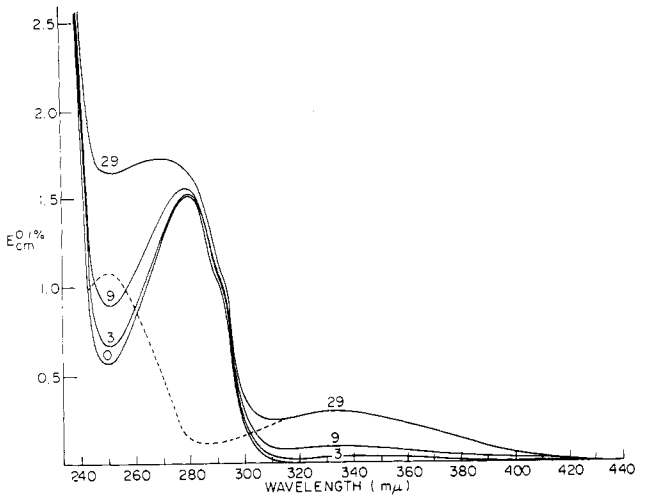

FIGURE 1: Ultraviolet absorption spectra of dansyl fibrinogen preparations of 1-mg/ml concentration. The numbers indicate the degree of labeling. Broken line: calculated difference curve between the spectrum of dansyl-F-29 and of native fibrinogen.

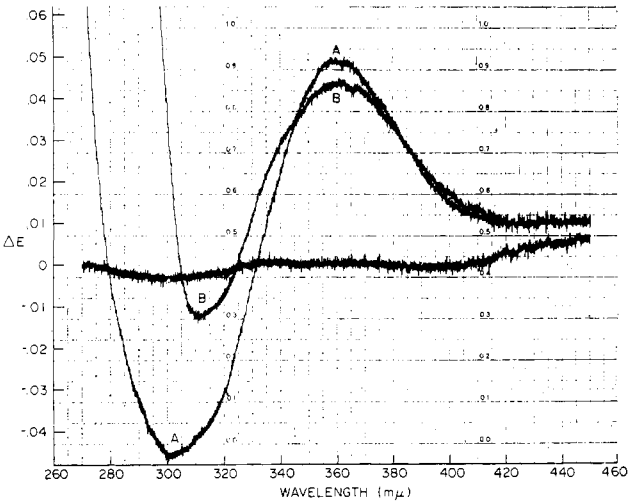

FIGURE 2: Difference spectrum of dansylglycine (A) and dansyl fibrinogen (B) obtained with 60% glycerol as perturbant: superimposed base line; dansylglycine concentration, 2.8×10^{-4} M; protein (dansyl-F-29) concentration, 1.6 mg/ml.

in very good agreement with the rhodamine B runs. The light intensity of the xenon source falls off drastically below 240 mμ, so that no reliable correction can be obtained below this point. In order to have an applicable correction factor, the excitation slit width should be the same in the above runs as in the actual experiments.

Emission spectra were corrected by a method suggested also by Melhuisch (1962). The variation with wavelength of the emission monochromator output–photomultiplier sensitivity was obtained by comparing the light source–excitation monochromator output determined previously, with the wavelength dependence of the photomultiplier response to the same light source. For this purpose a metallic 45° mirror, supplied with the instrument, was inserted in the cell holder. The excitation wavelength was changed continuously by the drive, while the emission monochromator was set manually to wavelengths in steps of 10 mμ. Each adjustment to the next step was performed while the excitation wavelength was at midpoint between steps. The recording had a sawtooth appearance, the top of the "teeth" corresponding to the phototube response when the wavelengths of the two monochromators were matched. The light intensity with this arrangement is orders of magnitude larger than with fluorescent light. To keep the excitation and emission slits at the same widths as in the experiments, the aperture of the photomultiplier was narrowed until the responses were on scale of the recorder with the lowest sensitivity of the instrument. The phototube responses at the same wavelength, obtained with the rhodamine B quantum counter were compared with the maximum of each tooth. The ratio of the two gives the correction factor by which the apparent emission at each wavelength has to be multiplied.

To avoid inner filter effects, optical densities of the fluorescing solutions were kept under 0.1 throughout the investigated wavelength range. Several dilutions were always run, both with excitation and emission spectra, to ascertain the concentration level below which the normalized fluorescent intensities remained constant at each wavelength. For a detailed description of the correction methods the reader is referred to Brand and Witholt (1967).

Fluorescent quantum yields were determined by the method of Parker and Rees (1960). A highly purified sample of dansylsulfonic acid, dissolved in 0.1 M $NaHCO_3$, served as standard, to which an absolute quantum yield of 0.36 was assigned (Chen, 1966). The latter value was confirmed by Turner and Brand (1968). The solutions were not purged by N_2, since this is not practicable with protein solutions because of their excessive foaming.

Fluorescent excited life times were measured with an apparatus supplied by TRW Instruments, El Segundo, Calif., which consisted of a Model 31A nanosecond flash unit and a Model 32A decay time computer, combined with a fluorescence detector unit and a dual-gun oscilloscope (Tektronix, Inc., Portland, Ore., Model 555). The arrangement and its mode of operation were described in some detail by Chen *et al.* (1967). The N_2-filled lamp was used at 2 kc, with a Corning 7-54 as primary and Corning 3-75 plus Wratten No. 8 as secondary filters. The apparatus was provided with a thermostated cell holder. Temperature was controlled to ±0.1° of the desired temperature.

Temperature in the cells was checked in all these experiments with a Tele-Thermometer from Yellow Springs Instrument Co., Yellow Springs, Ohio.

Results

Spectroscopical Properties of Dansyl Fibrinogen Conjugates. ULTRAVIOLET ABSORPTION SPECTRA. The spectra of a series of dansylated fibrinogen preparations are shown in Figure 1. The calculated difference between the highly labeled dansyl-F-29 preparation and the unlabeled material is shown by the broken line. This line coincides above 320 mμ with the actual spectrum of the labeled protein, since the unlabeled one has no absorption in this region. The difference curve shows the two characteristic peaks of the dansyl group at 250 and 335 mμ. The position of these is identical with that of ε-dansyllysine in methanol, however, their height ratio (3.7) is slightly higher than the one observed with dansylamino acids (3.1–3.3). Also, the ratio of absorbances at 280 and 335 mμ is slightly larger than with the model compounds (0.45 *vs.* 0.31–0.34). The reason for this is probably the insufficient correction for scattering of the dansylated sample.

TABLE I: Effect of Temperature and Glycerol Concentration on the Relative Quantum Efficiencies of a Low-Labeled and a High-Labeled Dansyl Fibrinogen Preparation.[a]

Per Cent Glycerol	Dansyl-F-3					Dansyl-F-27				
	Temperature, °C					Temperature, °C				
	5.4	14.1	22.6	30.1	39.0	6.6	11.8	21.0	30.2	38.5
0	1.118	1.087	1.000	0.970	0.861	1.152	1.075	1.000	0.906	0.845
10	1.243	1.193	1.078		0.909	1.215	1.164	1.126	1.000	
30	1.523	1.369	1.319	1.263	1.056	1.479	1.392	1.269		1.034
60	2.259	2.217	1.884	1.923	1.587	1.890	1.702	1.595	1.491	1.289

[a] Values were normalized with respect to the emission at 22.6°, or at 21.0°, in the absence of glycerol. Samples contained 0.1 M KCl–0.05 M phosphate buffer, pH 7.8, and varying amounts of glycerol. Protein concentration was 0.61 mg/ml with dansyl-F-3 and 0.102 mg/ml with dansyl-F-27; concentration with respect to dansyl groups was 6.2×10^{-6} M with dansyl-F-3 and 8.1×10^{-6} M with dansyl-F-27.

Figure 2 shows a comparison of the perturbation spectra of dansyl-F-27 and of dansylglycine, with 60% glycerol as the perturbing agent. The two spectra are similar above 320 mμ. Below this, the positive perturbation spectrum of the protein moiety is added to the perturbation spectrum of the dye, which results in an upward shift of the difference spectrum of the protein–dye conjugate with respect to the model compound. The data, when corrected for the difference in concentration of the dansyl residues in the two experiments, suggest 83% exposure of the dansyl residues of the conjugate to the perturbing agent.

FLUORESCENT EXCITATION SPECTRA. The corrected spectra of two dansylated fibrinogen preparations of different degrees of labeling are shown on Figure 3. The two maxima of the dye absorption are apparent at the 330- and 250-mμ region, together with an inflection at about 280 mμ, corresponding to the absorption peak of the native protein. The two normalized spectra are very similar, with only a small shift in the position of the 330-mμ region peak, toward longer wavelengths, with increase of labeling. The difference in height of the 250-mμ peak may not be significant in view of the large correction factors of this region.

FLUORESCENCE EMISSION SPECTRA. These are presented in Figure 4 for the same two conjugates as with the excitation spectra. The emission peak appears to be shifted to higher wavelengths with increase in the number of the attached dye molecules. Also, there is a shoulder at 490 mμ with dansyl-F-6, which is barely visible with the other preparation of higher degree of labeling.

The excitation and emission spectra of the conjugates will be discussed in more detail in connection with the fluorescence spectra of model compounds.

QUANTUM YIELDS. The emission spectrum of dansyl-F-3 and of dansyl-F-27 was determined at five different temperatures, in the absence and in the presence of three glycerol concentrations. The relative quantum efficiencies under these conditions were evaluated and are given in Table I. These are proportional to the areas of the corrected emission spectra at constant exciting energy. Each sample of a series contained the same amount of protein–dye conjugate, however, their absorption changed slightly under the various conditions because of the perturbation of the absorption

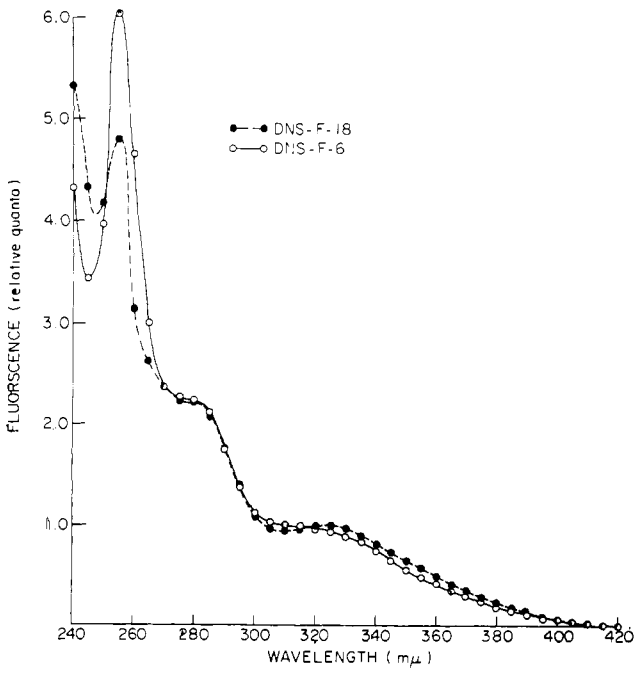

FIGURE 3: Corrected fluorescent excitation spectra of two dansyl fibrinogen preparations, normalized with respect to the 335-mμ dansyl absorption peak: protein concentration, 0.022 mg/ml and 0.0074 mg/ml, respectively.

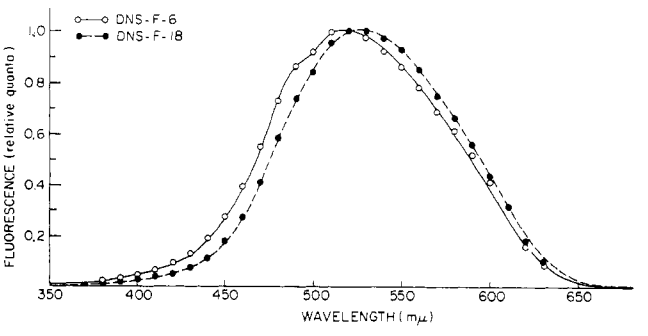

FIGURE 4: Corrected fluorescence emission spectra of two dansyl fibrinogen preparations, normalized with respect to the emission maximum: protein concentration with both solutions 6.1 mg/ml.

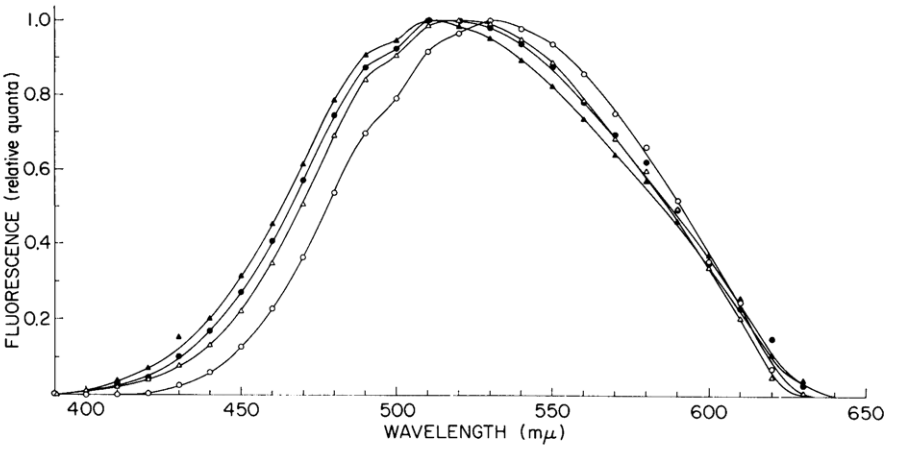

FIGURE 5: Corrected emission spectra of fibrinogen with low degree of labeling (dansyl-F-3), at low and high temperature, in the absence and in the presence of 60% glycerol, normalized with respect to the emission peak height: protein concentration, 0.6 mg/ml in 0.1 M KCl–0.05 M phosphate, pH 7.8; symbols, (○) 5.4°, 0% glycerol; (△) 39.0°, 0% glycerol; (●) 5.4°, 60% glycerol; (▲) 39.0°, 60% glycerol.

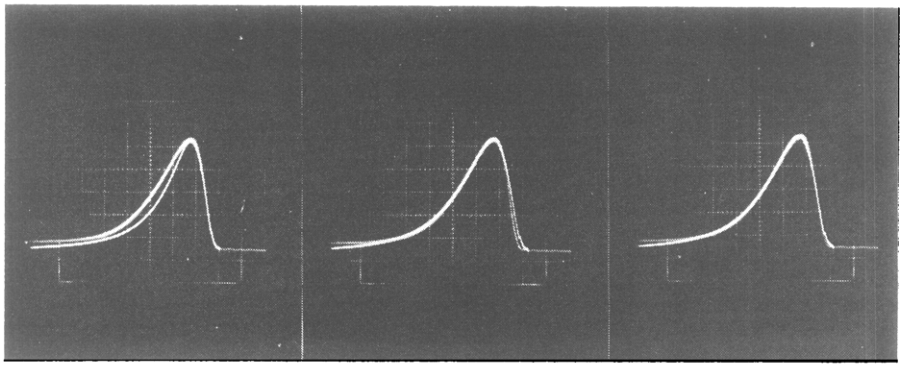

FIGURE 6: Oscilloscope tracings of the fluorescence decay of dansyl fibrinogen (dansyl-F-6); wider line fluorescence intensity, sharper line computed curve. Curves run from right to left. Left, match of the ascending limb; middle, of the descending limb; right, of both sides.

spectrum both by change in temperature and glycerol concentration. The correction factors for the effect of glycerol were evaluated from the difference spectrum with 60% glycerol (Figure 2), and for the effect of temperature from a difference spectrum between two samples of the same solution of dansyl-F-29, one kept at 8.8°, the other at 33.8°. It was assumed that the effects of both glycerol and temperature are linear. At 335 mμ the correction is 0.0586% per 1% of glycerol and −0.114% per degree Celsius.

The quantum efficiency increases with glycerol concentration and decreases with increase of temperature. The effects are very similar with both preparations, except for a higher effect of 60% glycerol on the efficiency of the low-labeled material. Plots of the relative quantum yields *vs.* temperature, at constant glycerol concentration, were linear within experimental error, with both preparations. Slopes were increasing with the glycerol concentration. Plots *vs.* glycerol concentration, at constant temperature, were linear with the high-labeled conjugate, but had a distinct upward curvature with the low-labeled one.

The corrected emission spectra of the two preparations (dansyl-F-3 and dansyl-F-27), at low and at high temperature, in the absence and in the presence of 60% glycerol, were normalized with respect to the fluorescent intensity at the maximum of the emission peak. The normalized spectra for dansyl-F-3 are shown on Figure 5. Both increased temperature and the presence of glycerol appear to shift the spectrum to lower wavelengths. Also, the shoulder at 490 mμ becomes relatively more important when the spectrum is perturbed by either increase of temperature, or by glycerol. The spectra of the more highly labeled material, which are not given here, show similar changes, but to a lesser extent, and the curves are equally spaced, without the initial abrupt shift caused by either temperature increase, or glycerol. The difference between the two preparations seems to reside mainly in the larger effect of these factors on the fluorescent species which emits with a maximum at 490 mμ and is relatively more abundant in the lower labeled conjugate.

In view of these shifts the variation in overall quantum yields cannot be accurately estimated from fluorescent intensity measurements at a single wavelength, especially, if the chosen emission wavelength is far from the maximum. The errors become even greater when the excitation wavelength is also far from the excitation maximum and no correction is made for the absorption changes mentioned in the previous paragraph, as for example, when dansyl groups are excited with the 366-mμ Hg line.

Absolute quantum yields are reported for preparations of various degrees of labeling in Table II. The fluorescence efficiency increases by about 40% as the number of bound dansyl groups increases from 3 to 27 per mole of fibrinogen.

FLUORESCENT EXCITED LIFE TIMES. It was not possible to match exactly the fluorescence signal by the computed curve. This indication of heterogeneity is shown on the oscillograph

TABLE II: Quantum Efficiencies of Dansylated Fibrinogen Preparations and of Some Model Compounds.

Material	Quantum Efficiency	
	Obsd	Calcd
Model Compounds		
ε-Dansyllysine	0.325	
O-Dansyltyrosine	0.045	
Didansyltyrosine	0.116	
Dansylated Fibrinogens		
Dansyl-F-4	0.109	0.095
Dansyl-F-9	0.126	0.135
Dansyl-F-18	0.151	0.157
Dansyl-F-27	0.156	0.200

[a] Quantum efficiencies were determined relative to dansylsulfonic acid in 0.1 M $NaHCO_3$ (Chen, 1966), to which an absolute quantum yield of 0.36 was assigned. The calculated values are based on the efficiencies of the model compounds and their proportion in the dansylated fibrinogens.

tracings of Figure 6, obtained with dansyl-F-6. A good fit to either the ascending or to the descending limb could be obtained, but not to both at the same time. Matching the ascending side gave a shorter decay time than the other side, and a match of both sides, in the best possible way, gave a value intermediate between the former two. The match became better as the number of bound dansyl residues increased, approaching a homogeneous appearance with dansyl-F-27.

Some of these data, obtained with a few preparations, are listed in Table III. The variation of the "average" excited lifetime with temperature and with glycerol concentration is shown in Figure 7. A linear relationship with temperature is apparent, and the excited lifetime becomes longer with increase in glycerol concentration. Also, the high-labeled conjugate has a longer decay time.

Model Compounds. SPECTROSCOPIC PROPERTIES. Since the fluorescent residues of the dansylated fibrinogen preparations

TABLE III: Excited Lifetime Measurements on Several Dansyl Fibrinogen Preparations.[a]

Preparation	Excited Lifetime (nsec)		
	Ascending[b]	Descending[b]	Average[b]
Dansyl-F-3[c]	8.9	14.5	12.6
Dansyl-F-3[c]	9.9	14.0	12.2
Dansyl-F-9	7.4	14.3	13.1
Dansyl-F-27	15.3	15.9	15.4

[a] Solvent was 0.15 M KCl–0.05 M phosphate, pH 7.8. Protein concentration was 0.5 mg/ml, temperature 20.° [b] The column headings refer to matching the experimental curve with the computed one on the ascending, or descending limb, or on both limbs in the best possible way. [c] Two different preparations of approximately the same degree of labeling.

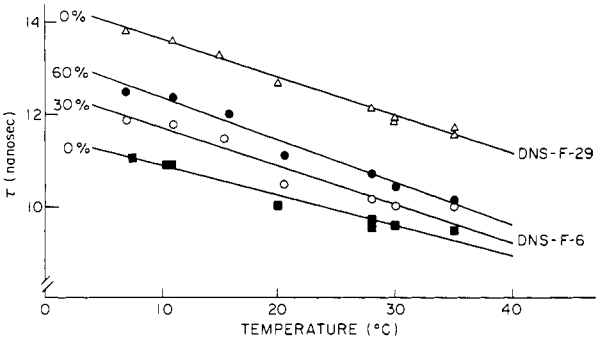

FIGURE 7: Excited lifetimes of two dansyl fibrinogen preparations as a function of temperature and glycerol concentration. Values were obtained by matching both limbs of the tracings (average values, as described in the text).

are of three different types, it was of interest to investigate the flourescent properties of these in their isolated state. Figure 8 shows the absorption and corrected excitation spectra of ε-dansyllysine. The two characteristic maxima are in their normal positions and the normalized absorption and excitation spectra coincide over the whole spectral range. The small difference at the low-wavelength end is probably a reflection of the inaccuracy of the correction of the excitation spectrum in this range.

An entirely different picture is apparent on Figure 9, which was obtained with *O*-dansyltyrosine. The absorption and the excitation spectra are not superimposed. The longer wavelength absorption peak is shifted to 349 mμ and the excitation peak in the opposite direction, to 326 mμ. The 250-mμ peak is similarly affected, but to a much lesser extent. The spectra were normalized with respect to the height of their peak in the 330-mμ region. However, this resulted in stronger emission than absorption between 280 and 335 mμ. Since this is not possible, the normalization should have been performed with respect to the latter spectral region. This would have reduced the height of the excitation spectrum by about 50%.

The peculiar behavior of *O*-dansyltyrosine is obviously the result of the interaction of the phenolic and naphthalenic chromophores. One possibility would be an equilibrium

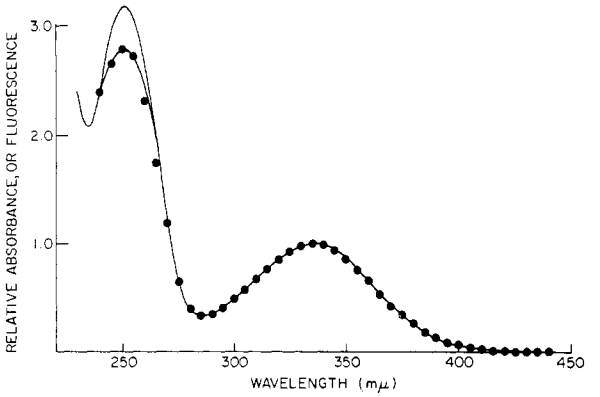

FIGURE 8: Absorption and corrected excitation spectrum of ε-dansyllysine in methanolic solution: continuous line, absorption; filled circles, fluorescence intensity, both normalized with respect to the maximum in the 330-mμ region; concentration for fluorescence measurements, 10^{-6} M.

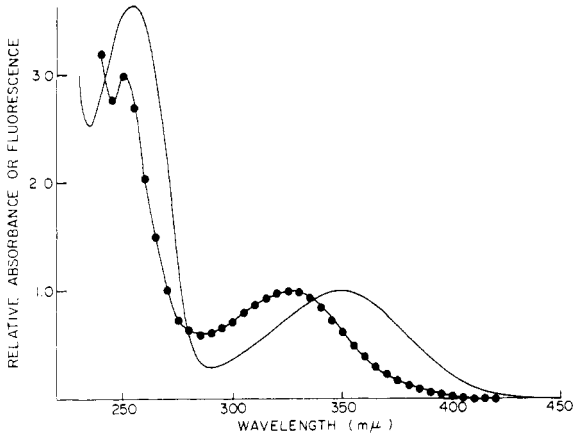

FIGURE 9: Absorption and emission spectrum of *O*-dansyltyrosine. Same conditions as for Figure 8.

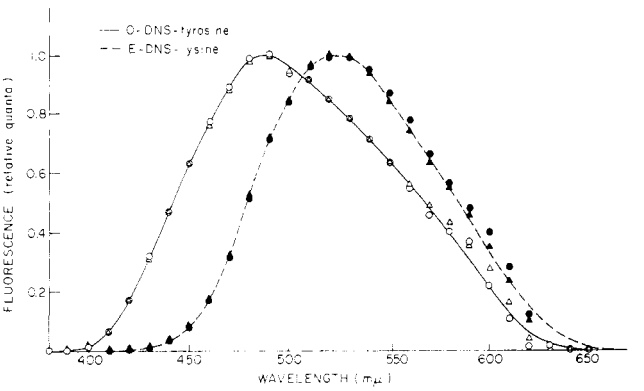

FIGURE 10: Corrected emission spectra of ε-dansyllysine and *O*-dansyltyrosine, dissolved in methanol, normalized with respect to their peak heights, each run at two different concentrations: (▲) 2×10^{-5} M, (●) 4×10^{-5} M, (△) 10^{-4} M, (○) 4×10^{-6} M.

between a fluorescent and a nonfluorescent state of the molecule, which may occur either in the ground state, or the first excited state. The transition of the fluorescent species would have a slightly higher energy, *i.e.*, the absorption would be situated at lower wavelengths, with the equilibrium favoring the nonfluorescent variety, as evidenced by the low quantum yield of the fluorescence, which will be described subsequently. Whether or not this hypothesis is the correct one, and the internal mechanism associated with it, cannot be established with the present data.

The emission spectra of both compounds are presented on Figure 10. The maximum of ε-dansyllysine is situated at approximately 520 mμ, that of *O*-dansyltyrosine at 490 mμ. Their quantum yields are given in Table II. The tyrosine derivative has an efficiency which is only about 14% of that of ε-dansyllysine. *O*-Dansyltyrosine is a very unstable compound. This is evidenced by the appearance of new spots on thin-layer chromatography of its methanolic solution, even if this was stored at −15° in the dark. A photochemical reaction also is present. The height of the emission peak increases by about 20% with each successive scan of about 5.5-min duration. Whether or not this phenomenon can be correlated with the appearance of breakdown products was not investigated, but it certainly suggests that the true quantum efficiency of *O*-dansyltyrosine might be even lower than the one determined in this work.

Because didansyltyrosine is quantitatively an unimportant component in dansyl fibrinogens, its spectra are not given here. They occupy an intermediate position between that of ε-dansyllysine and *O*-dansyltyrosine, with a preponderance of the former. Thus, as expected, the dansyl group attached to the α-NH_2 group of the molecule behaves more or less normally. The discrepancy between absorption and excitation spectra is present, but to a much lesser degree than with *O*-dansyltyrosine. Emission maximum is at 520 mμ, which is the same as for ε-dansyllysine, and its quantum yield is roughly one-half of that of the latter.

Discussion

Spectroscopic Aspects. The two different kinds of anchor groups of the dansyl residues are manifest in the spectroscopic properties of the conjugates and all the peculiarities observed with the latter can be rationalized on this basis. When the absorption spectra are normalized with respect to the peak height in the 330-mμ region a shift toward shorter wavelengths is observed with the increase of the number of bound dansyl residues. At the same time the excitation and emission peaks are displaced in the opposite direction (Figures 3 and 4). This is a consequence of the predominance of dansyltyrosine residues in the low-labeled material, with an imprint of their characteristics on the resulting spectra, and a shift to the spectral features of ε-dansyllysine, as the proportion of the latter increases.

The overall quantum yield of the conjugates increases with the degree of labeling (see Table II), and so does the excited lifetime (Figure 7). These have the same explanation as the aforementioned changes in the spectral properties. The theoretical proportionality between quantum yield and excited lifetime is approximately satisfied with the variations with temperature. The quantum yield ratio for the 10–40° range for dansyl-F-3, read on plots of the data of Table I, is 1.25, whereas the ratio of the excited lifetimes for dansyl-F-6, for the same temperature interval from Figure 7, is 1.22. The difference is probably within the experimental errors, especially considering that the data were obtained on two different preparations. However, a much larger discrepancy is evident for the effect of glycerol. The data mentioned above give a 1.87-fold increase in quantum yield, but only 1.12-fold increase for the excited lifetime, when the glycerol concentration is increased from zero to 60% at 20°. This comparison may not be justified entirely because the excited lifetime increases linearily, whereas the quantum yield increases hyperbolically with the glycerol concentration. But, a comparison of the initial slopes also reveals a large difference. These were 2×10^{-3} for the relative increase of excited lifetimes and 7.5×10^{-3} for that of the quantum yields for 1% of glycerol. With dansyl-F-27 the quantum yield plot was linear up to 60% of glycerol and the increment was even higher, 11.6×10^{-3} for 1% of glycerol. Unfortunately, data for the excited lifetime variation with glycerol concentration were not obtained with this preparation. Very likely the discrepancy is caused by the differences in the nature of the "averages" determined in the two types of experiments, but a more complex effect of glycerol on the fluorescence phenomena is also possible.

The theoretical relationship appears to hold again for the efficiency and excited lifetime variations with the number of bound residues in the absence of glycerol. This is evident from comparison of the data of Table II and Figure 7, though

the excited lifetime data are not numerous enough for accurate evaluation.

Nature of the Environment of the Attached Dye Molecules. Fluorescent quantum yields are a very sensitive indicator of the polarity of the medium (Stryer, 1965; McClure and Edelman, 1966). The extensive investigations of Turner and Brand (1968) have established the relationship between these two quantities for some *N*-arylaminonaphthalenesulfonates and applied it to the determination of the polarity of binding sites for the dyes in a large number of proteins. A similar dependence on polarity was observed by Chen (1967) for the fluorescence of some dansylamino acids and probably this is true also for the dansylamino acids investigated in this work. An attempt was made to calculate the overall quantum yields of dansyl conjugates of fibrinogen from the relative proportion of their fluorescent residues, as given in Table III of the previous paper, and the quantum yields of these in the free state and in methanol, as listed in Table II. The results of these calculations are included in Table II and it is seen that they are in fairly good agreement with the experimental data. This would indicate that methanol is of approximately the same polarity as the environment in which the dansyl residues find themselves when bound to fibrinogen. The same conclusion holds for the large majority of the proteins investigated by Turner and Brand, even though noncovalently bound dyes, of different but related structures were used. Methanol is a fairly polar molecule, and this finding is probably not at odds with the 83% exposure of the fluorophores to the perturbing molecules and presumably to water also.

Energy Transfer. The excitation spectra of dansyl fibrinogens show a pronounced shoulder at about 280 mμ (Figure 3). In this region, the excitation spectrum of dansylamino acids exhibits a minimum, therefore, energy transfer from the protein chromophores absorbing in this region must occur. These relationships are illustrated in Figure 11, which shows for dansyl-F-18 the absorption and excitation spectrum, both normalized with respect to the 335-mμ peak, together with the excitation spectrum of ε-dansyllysine, also normalized at the above wavelength. The difference between the excitation spectra of the conjugate and of the model compound corresponds to the energy transferred from the protein fabric to the fluorophore. It shows a maximum at about 282 mμ, which is close to the absorption maximum of the protein. Uncertainties in the position and height of the short-wavelength peak of the excitation spectra of both dansyl fibrinogen and of ε-dansyllysine are the probable reason for the deviation of this difference spectrum from that of the native protein below about 265 mμ. The ratio of the height of the difference peak to the absorption of the protein–dye conjugate at this wavelength gives the fraction of energy transferred. With dansyl-F-18 the efficiency of the transfer is 19%. The normalized excitation spectrum of dansyl-F-9 is virtually identical in this region with that of dansyl-F-18. Therefore, the energy transferred per dansyl residue must be the same with both conjugates. However, the total absorption at 280 mμ per dansyl residue is about 3 times larger for the lower labeled conjugate, which gives for the latter a transfer efficiency of only 6%. Since there are a large number of donor and acceptor groups involved in the transfer process, it is not possible to calculate from the data the distances between participating groups. The fact that the energy transferred to

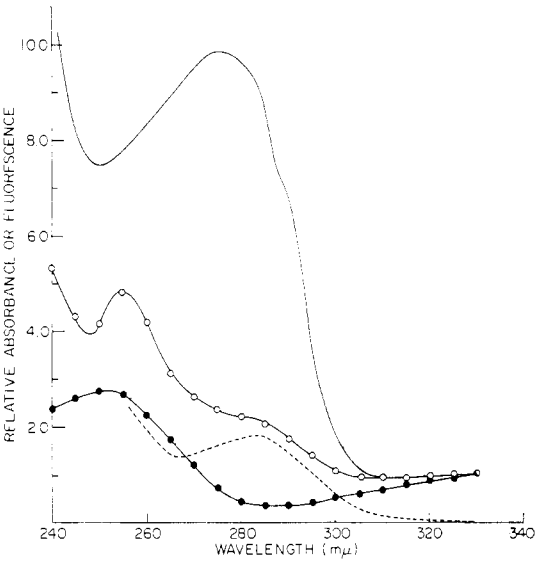

FIGURE 11: Energy transfer in dansyl fibrinogen (dansyl-F-18): continuous line, absorption spectrum; empty circles, excitation spectrum of dansyl fibrinogen; filled circles, excitation spectrum of ε-dansyllysine. All three curves normalized with respect to their peak heights at 335 mμ. Broken line, difference between the excitation curves of dansyl fibrinogen and of ε-dansyllysine, corresponding to the energy transfer.

any one group was about the same in the two conjugates of different degrees of labeling would indicate that the environment from which transfer occurs is more or less the same, regardless of the number of acceptors, at least within the range of labeling defined by the two conjugates investigated. Energy transfer within dansyl conjugates of fibrinogen also was shown to occur by Slayter and Hall (1964). This was inferred from a powerful protection of the protein against denaturation by ultraviolet irradiation by means of conjugation with dansyl groups. They also estimated from spectroscopical measurements, similar to the ones used in this study, a transfer efficiency of 13% for a conjugate of 7 dansyl moles per mole of fibrinogen. In view of the numerous corrections involved and different techniques used, this figure is in reasonable agreement with the ones reported in this paper.

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