Study of the Dansylation of Bovine Fibrinogen. Some General Properties of the Conjugates and Identification of the Binding Sites for the Dansyl Groups*

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ABSTRACT: A series of dansyl conjugates of bovine fibrinogen were prepared with the degree of labeling increasing from 3 to 36 moles of dye per mole of protein. The first 6–8 moles bound have a very small effect on the general physicochemical properties of the molecule (salting out, sedimentation, optical rotation), whereas the latter ones bring about substantial changes in these. The binding sites for the dansyl residues were determined by thin-layer chromatography of acid hydrolysates: *O*-(1-dimethylaminonaphthalene-5-sulfonyl)-tyrosine, di-(1-dimethylaminonaphthalene-5-sulfonyl)tyrosine, and ε-(1-dimethylaminonaphthalene-5-sulfonyl)lysine were identified, together with dansyl sulfonic acid, which is probably a hydrolytic decomposition product of the first two. At low degrees of labeling the ratio of dansylated tyrosines to dansylated lysine residues is between 3 and 4. With increase of labeling this ratio approaches unity. The data suggest that the dansyl groups are not distributed randomly, but are bound preferentially to a few specific sites of the protein.

1-Dimethylaminonaphthalene-5-sulfonyl[1] conjugates of native proteins have been used extensively for various purposes, taking advantage of their fluorescent properties. It was generally assumed that the dye molecules are attached randomly and principally to the ε-amino residues of the proteins (Weber, 1952). There has been no attempt to establish directly the points of attachment, except in one case, when Hartley and Massey (1956) found that in chymotrypsin of very low degree of labeling the dansyl group is bound to a specific histidine residue. Later Chen (1968a) called attention to the fact that fluorescent properties of bovine serum albumin conjugates showed heterogeneity and this could be the result of variations in the residues to which the dye is bound. Dansyl chloride reacts and forms stable compounds with a variety of side chains, beside the ε-amino groups (Gray, 1967). These are available also in the native protein and, therefore, it is expected that the label will be found not only on the ε-amino, but on other reactive groups too.

In the present work experiments were carried out with dansyl conjugates of bovine fibrinogen of different degrees of labeling. Various chemical techniques were used to determine the distribution of the dye groups among the possible sites of the molecule. Naturally, without knowing the primary structure of the chains, the position of these residues cannot be pinpointed. Nevertheless, these studies revealed that there are a fairly limited number of specific sites involved in the reaction, *i.e.*, the label is not uniformly distributed among the susceptible sites. Also, beside the ε-amino groups, a substantial fraction of the dye was bound to the phenolic groups of tyrosine residues.

The large hydrophobic groups attached to the surface of the molecule have an effect on the general properties of the molecule and these were also investigated to some extent.

Materials and Methods

Preparation of the Fluorescent Conjugates. Bovine fibrinogen, obtained from Armour Pharmaceutical Co., Kankakee, Ill., Lot No. B 7907, was purified by Laki's (1951) method. The dansyl conjugates were prepared according to Rinderknecht (1962), adding dansyl chloride adsorbed on Celite (dye content 9.8%, as supplied by Mann Research Laboratories, New York, N. Y.) to a 1% fibrinogen solution in 0.3 M KCl, 0.1 M phosphate buffer of pH 7.8, in a 1:1 weight ratio of protein to Celite–dye adsorbate. The mixture was placed in an ice bath and stirred continuously. For higher degrees of labeling the reaction was performed in carbonate–bicarbonate buffers with pH values ranging from 8.2 to 9.8. The reaction was stopped after 1 to 18 hr by centrifuging out the dansyl chloride–Celite from the protein solution. The free dye was removed chromatographically in a Sephadex G-25 column, using 0.05 M phosphate buffer (pH 7.8)–0.1 M KCl as eluent. The samples collected from the column were monitored at 280 and 335 mμ. The elution diagrams showed in all cases a sharp peak that contained most of the protein, followed by a broad peak with a high absorption at 335 mμ and about half as much at 280 mμ. The amount of free dye in the samples was estimated by fluorimetry. The labeled protein was precipitated with 20% acetone and the supernatant was used for the estimation. The eluate in the first peak was free of uncoupled dye and it was collected for subsequent experiments.

Protein concentration and the degree of labeling were estimated from the absorption of the conjugates at 280 and 335 mμ by a procedure similar to that described by White (1964); the extinction coefficients used were $E^{1\%}_{cm}$ 15.06 for fibrinogen (Mihalyi, 1968), and ϵ^{M}_{cm} 3400 for the dansyl group (Chen, 1968b), at their respective maxima. For the concentration determination, the absorption at 280 mμ was corrected for turbidity and contribution of the dye absorption at this wavelength. For the former, the absorption at 450 mμ, where neither protein nor dye absorb, was subtracted from the 280-mμ absorption. Spectra of several dansyl-amino acids dissolved in methanol (α-dansyl derivatives of aliphatic amino acids and ε-dansyllysine) revealed a maximum

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

TABLE I: Reaction Conditions and Degrees of Labeling of Dansyl Fibrinogen Conjugates.

Designation	Reaction Conditions: Time (hr)	pH	Buffer	Degree of Labeling (moles of Dansyl/mole of Fibrinogen)	
	18	6.8	Phosphate	3.6	
	20	7.5	Phosphate	3.4	
Dansyl-F-3	1	7.8	Phosphate	3.5	Average 3.3
	1.4	7.8	Phosphate	3.1	
	20	7.8	Phosphate	2.8	
Dansyl-F-6	18	8.2	Carbonate	5.8	
Dansyl-F-9	20	8.5	Carbonate	8.7	
Dansyl-F-18	20	9.1	Carbonate	18.2	
Dansyl-F-27	20	9.6	Carbonate	27.0	
Dansyl-F-29	16.5	9.6	Carbonate	28.9	
Dansyl-F-36	5.5	9.8	Carbonate	30.9[a]	
				36.0[b]	

[a] Soluble at pH 7.8. [b] Insoluble at pH 7.8, soluble at pH 9.2.

at approximately the same position as that of the protein-bound dye and a minimum near 280 mμ. The average of their ratios of absorbancies at 280 and 335 mμ was 0.34, and this figure was used to evaluate, from the height of the dye peak, the contribution of the dye to the 280-mμ absorption. The degree of labeling was calculated assuming a molecular weight of 340,000 for native fibrinogen.

Clottability of the samples was estimated by the procedure of Laki (1951), using Thrombin, Topical, of bovine origin, obtained from Parke, Davis and Co., Detroit, Mich., and purified by the method of Rasmussen (1955).

Reagents. Dansylamino acids purchased from Calbiochem, Los Angeles, Calif., proved of sufficient purity, except the didansyl-L-tyrosine, which was heavily contaminated and had to be purified by chromatography. Concentration of the dansylamino acids was estimated by spectrophotometry, assuming that all have the same maximum molar extinction coefficient of 4300, in methanolic solution, irrespective of the position of the maximum. This approximation is justified in view of the similar values reported by Weber (1952) for dansylsulfonic acid in water and dansylamide in 60% ethanol. The methanolic solutions of the dansyl derivatives of tyrosine are unstable even at −15° and in the dark. After a few weeks a large number of spots appear on thin-layer chromatography of solutions which originally had only one spot present. ε-Dansyllysine did not show signs of decomposition.

Chemical Methods. PEPTIDE MAPS OF COMPLETE TRYPTIC DIGESTS (FINGERPRINTS). These were obtained by high-voltage electrophoresis–chromatography using the procedure described by Custer (1969). The maps were scanned in the dark with a long-wavelength ultraviolet lamp, and the fluorescent spots outlined. After this, they were stained with ninhydrin using the method of Easley (1965).

IDENTIFICATION AND QUANTITATION OF THE DANSYL DERIVATIVES. The labeled protein was precipitated with 3 volumes of ether–methanol (1:2), washed with 50% aqueous methanol and absolute methanol, and dried. The dansyl derivatives of the amino acids were liberated from the protein by hydrolysis at 108° for 4 and 18 hr, in 6 N HCl and N_2 atmosphere (Gros and Labouesse, 1969). The hydrolysates were dried *in vacuo*, dissolved in methanol, and spotted on polyamide layers supplied by Cheng Chin Trading Co., Taipei, Taiwan. Chromatography in two dimensions was performed according to Woods and Wang (1967), as modified by Hartley (R. Hartley, personal communication). Solvent 1 was 0.15 M aqueous ammonium hydroxide and solvent 2 was heptane–1-butanol–formic acid (10:10:1, v/v). The fluorescent spots were identified by comparison to standards on the basis of their positions and their distinct color differences. The sulfonic acid spots were eluted with 0.1 M $NaHCO_3$, the others with methanol and estimated quantitatively by fluorimetry. Since the fluorescent properties of the derivatives vary considerably among each other, each component was estimated against its own standard, at the optimum excitation and emission conditions. The solutions of the tyrosine derivatives have a very low and unstable fluorescent intensity. They were evaporated to dryness, dissolved in 2 N KOH, hydrolyzed at 100° for 30 min to the free sulfonate, and estimated as such in the alkaline solution.

The dansyl derivatives were also quantitated directly by scanning the thin-layer plates with the chromatogram spectrophotometer, manufactured by Zeiss, Oberkochen, Germany (Jork, 1968). Excitation was at 335 mμ, with a slit of 2 mm. The curves were integrated by planimetry and their area was compared with that of standards obtained under identical conditions.

The estimates obtained by both methods were corrected for destruction during hydrolysis with the factors given by Gros and Labouesse (1969).

Physical Methods. Sedimentation studies were performed in the Spinco Model E analytical ultracentrifuge. Optical rotatory dispersion was determined in a Cary Model 60 recording spectropolarimeter. Fluorescence intensity was measured in a Hitachi-Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A coupled to a Model QPD 33 recorder of the same manufacturer.

Results

Degree of Labeling. The reaction conditions and degrees of labeling of a series of preparations are summarized in Table I. It appears that the pH has a more profound influence on the number of bound dye molecules than the reaction time, and at each pH there is a limiting number, beyond which the labeling does not proceed easily. This number increases sharply with pH and this is also shown in Figure 1.

Dansyl chloride is rapidly decomposed by water. Therefore, it seemed possible that the apparent time independence of the reaction was caused by a fast inactivation of the reagent. However, control experiments showed that after 1 hr of incubation at 0° and either pH 7.1 or 9.2, the amount of dansyl chloride recovered from the Celite suspension was 98 and 90%, respectively, of the amount originally present. At 25° and pH 7.1 the recovery decreased to 76%. Thus, the reagent adsorbed on Celite is fairly stable, whereas according to Gros and Labouesse (1969) it decomposes with a half-life time of 6.6 min in 0.1 mM solution at 15° and pH 7–9. These estimates were made spectrophotometrically using the method of the above authors, after the Celite was centrifuged off and the dansyl chloride extracted with absolute ethanol.

The amount of dansyl chloride in the reaction mixtures corresponded to 123 moles per mole of fibrinogen. Of this

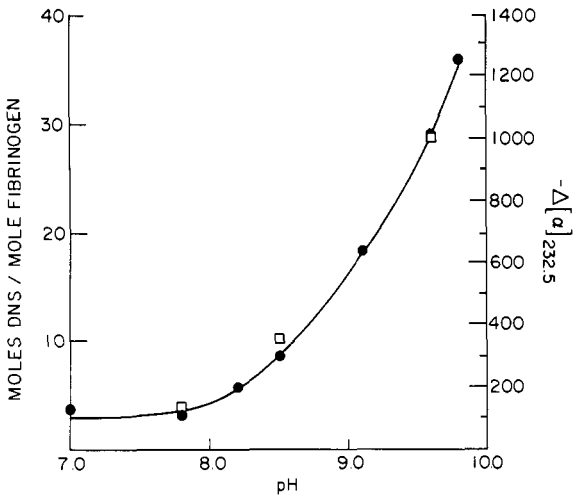

FIGURE 1: Increase of the degree of labeling with the pH of the reaction medium and correlation of the former with the change in optical rotation at the 232.5-mμ trough: Degree of labeling (●), $-\Delta[\alpha]_{232.5}$ (□).

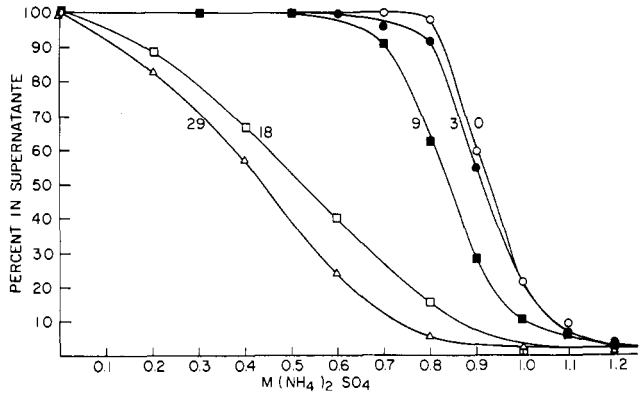

FIGURE 2: Salting-out curves of dansyl fibrinogen preparations of various degrees of labeling. The number next to each curve indicates the number of dansyl groups per mole of fibrinogen. The initial fibrinogen concentration was 0.44 mg/ml, in 0.05 M phosphate, pH 6.7, and the molar concentration of $(NH_4)_2SO_4$ was as indicated on the abscissa.

36 moles, 29% of the total, was bound in the highest labeled preparation. The high efficiency of the reaction also indicates the stability of the reagent in the Rinderknecht procedure.

The above experiments suggest that, at least at low pH, there are a few specific sites on the molecule which are labeled very fast. This contention was substantiated by an experiment in which after 1-hr reaction with fibrinogen at 0° and pH 7.1 the Celite–dansyl chloride was removed, replaced with a fresh charge, and this allowed to react for another hour. The amount of labeling after the second treatment increased by only 13% over that of the first one. The material with more than 20 moles of dye molecules per mole of fibrinogen is fairly insoluble in the pH 7.8 solvent used for elution from the Sephadex column. Shortly after elution a precipitate forms in the initially clear eluate, which comprises the larger part of the material. The precipitate is soluble at pH 9.2 and has only a slightly higher degree of labeling than the fraction remaining in solution. Thus, the precipitation achieved very little fractionation with respect to labeling and is more the reflection of the reduction of solubility of a fairly homogeneous material.

In the following, the dansyl fibrinogen conjugates will be denoted by dansyl-F-*n*, *n*, indicating the number of moles of dye bound per mole of fibrinogen.

General Properties of the Dansyl Fibrinogen Conjugates. SEDIMENTATION IN THE ULTRACENTRIFUGE. Dansyl-F-6 at 8.2-mg/ml concentration, at pH 7.8, 0.3 ionic strength and 20°, sedimented as a sharp symmetrical boundary, preceded by a small faster peak. The $s_{20,w}$ of the two peaks was 7.1 and 10.6 S, respectively, and their relative areas were 0.91 and 0.09. The high-labeled dansyl-F-29 preparation at 4.0-mg/ml concentration, at pH 9.2, 0.2 ionic strength and 20°, showed a sharper, slower, and a more diffused, faster component. Their $s_{20,w}$ was 7.9 and 12.1 S and the irrelative areas were 0.60 and 0.40.

The sedimentation rate of the slower sedimenting material, in both preparations, was approximately the same as that of unlabeled fibrinogen of similar concentration. The amount of aggregated material with the lower labeling was also about the same as that seen in usual native fibrinogen preparations. The high-labeled material, on the other hand, was to a large extent aggregated, in spite of the higher pH used in this experiment.

SALTING OUT. The salting-out characteristics of native and labeled fibrinogens is shown on Figure 2. The first 3 dye molecules appear to have very little effect, further ones shift the salting-out curves to much lower salt concentrations. The curve reported previously (Johnson and Mihalyi, 1965), with 11 bound dansyl groups, fits well with the curves reported here, indicating that the preparations obtained by the two different procedures have the same properties. Figure 2, and more explicitly a plot of the salt concentration at half-precipitation against the number of bound dye molecules, shows a fairly sharp transition between the more and the less soluble derivatives of the protein, centered at about 14 dye molecules bound. The broad precipitation zone with the highly labeled material may be a reflection of heterogeneity.

OPTICAL ROTATORY DISPERSION. The optical rotatory dispersion parameters of the preparations are given in Table II. The Yang and Doty (1957) and Moffitt and Yang (1956) plots were linear between 600 and 300 mμ and showed a pronounced curvature below this range both with the native and with the labeled materials. The λ_0 and b_0 values of the native protein are slightly lower than the ones reported previously (Mihalyi, 1965), probably because of the wider range and higher accuracy of the present determinations. Figure 1 shows that there is a very good correlation between the number of dye molecules bound and the change in the optical rotatory parameters. The helical content, if one wishes to convert the rotatory parameters into this quantity, shows a gradual decrease with the extent of labeling. Thus, with the more sensitive parameters a definite change of the protein conformation can be detected, changes which were not apparent when only $[\alpha]_D$ was determined on a low-labeled material (Johnson and Mihalyi, 1965). Since the labeling was performed at increasingly higher pH values, there was a possibility that the pH increase caused a conformation change which favors the binding of dansyl molecules and when these were bound the conformation change was stabilized. Experiments, however, showed that the $[\alpha]_{232.5}$ value was unchanged with native fibrinogen between pH 7.1 and 10.0. Thus, the observed conformation change follows the binding of the dansyl molecules.

Chemical Properties of the Dansyl Fibrinogens. THIN-LAYER CHROMATOGRAPHY OF THE HYDROLYSATES. Thin-layer chro-

TABLE II: Optical Rotatory Dispersion Parameters of Dansyl Fibrinogens.

Preparation	$[\alpha]_{589}$	λ_c	Helical Content	b_0	Helical Content	$[\alpha]_{232.5}$	Helical Content
Native	−49.3	250.3	28	−186	30	−5885	27
Dansyl-F-3	−48.6	250.3	28	−175	28	−5748	26
Dansyl-F-9	−48.8	250.3	28	−157	25	−5531	24
Dansyl-F-29	−46.3	243.4	23	−134	21	−4883	20

TABLE III: Quantitative Estimation of the Dansyl Derivatives in Acid Hydrolysates of the Labeled Fibrinogen Preparations.

Compound	Preparation					
	Dansyl-F-3	Dansyl-F-6		Dansyl-F-18		Dansyl-F-27
	Direct[a]	Direct[a]	Eluate[b]	Direct[a]	Eluate[b]	Direct[a]
Dansylsulfonic acid	1.3	1.3	3.0	1.0	4.3	2.4
ϵ-Dansyllysine	0.3	0.8	0.7	5.2	5.9	10.9
O-Dansyltyrosine	0.9		1.8	6.8	6.5	13.0
Didansyltyrosine	0.5		0.6	1.2	0.7	1.8
Sum	3.0		6.1	14.2	17.4	28.1
Total from spectral data	3.4	5.7		18.2		27.0
O-Dansyltyrosine + didansyltyrosine/ ϵ-dansyllysine	4.7		3.4	1.5	1.2	1.3

[a] Estimated directly on the thin-layer chromatograms with the Zeiss chromatogram spectrophotometer. [b] Estimated by fluorimetry on eluates from thin-layer chromatograms. Data are expressed as moles of dansyl derivatives per 340,000 g of fibrinogen, the assumed molecular weight of the protein.

matograms showed, regardless of the degree of labeling, the presence of four spots. These were identified as dansylsulfonic acid, ϵ-dansyllysine, *O*-dansyltyrosine, and didansyltyrosine. Not even traces of dansylglutamic acid, which should arise from the N terminus of the γ chain, were present in any of the preparations. In Figure 3 the chromatogram of the hydrolysate of dansyl-F-18 is compared with that of a mixture of corresponding standard dansylamino acids. The quantitative estimates of the fluorescent derivatives, both with the elution and the direct reading method, are listed in Table III. The two methods gave results in satisfactory agreement with each other and with the total number of dyes determined spectrophotometrically. Only the estimates of dansylsulfonic acid were substantially higher in the eluates than with the direct determination. The 4-hr hydrolysis time recommended by Gros and Labouesse (1969) gave 25–35% lower values than the 18-hr time, consequently, the longer time was used throughout this work.

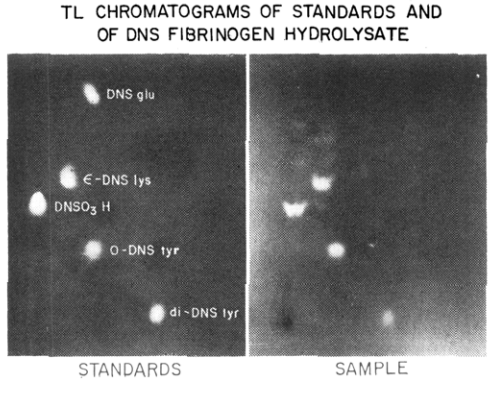

FIGURE 3: Thin-layer chromatogram of a mixture of standard dansylamino acids (left side) and of an acid hydrolysate of dansyl fibrinogen (dansyl-F-18) (right side). The amount in one spot in the standards is approximately 5 nmoles. The amount in one spot of the sample is 0.25–1.2 nmoles: origin, left lower corner; first dimension, vertical, 0.15 M NH_4OH; second dimension, horizontal, heptane–1-butanol–formic acid, 10:10:1.

FINGERPRINTS. The peptide maps stained with ninhydrin were practically identical with that of native fibrinogen. Within the limits of confidence inherent with such a complex map, all the spots were present which are characteristic for native fibrinogen and no new ninhydrin spots appeared. The number of fluorescent spots were slightly higher with high-labeled protein and the spots were situated outside of the positions occupied by the ninhydrin spots of native fibrinogen. Their chromatographic mobility appears to be larger than with the spots of the native material. In all, there were about four fluorescent spots which appeared in nearly all the samples, regardless of the degree of labeling and another two, which were more evident with higher degrees of labeling. A variable number of spots ranging from 6 to 10 also appeared, but their intensity and reproducibility were much lower. Figure 4 shows a ninhydrin-stained map of dansyl-F-27 on which the fluorescent spots are outlined. The fluorescent spots disappear

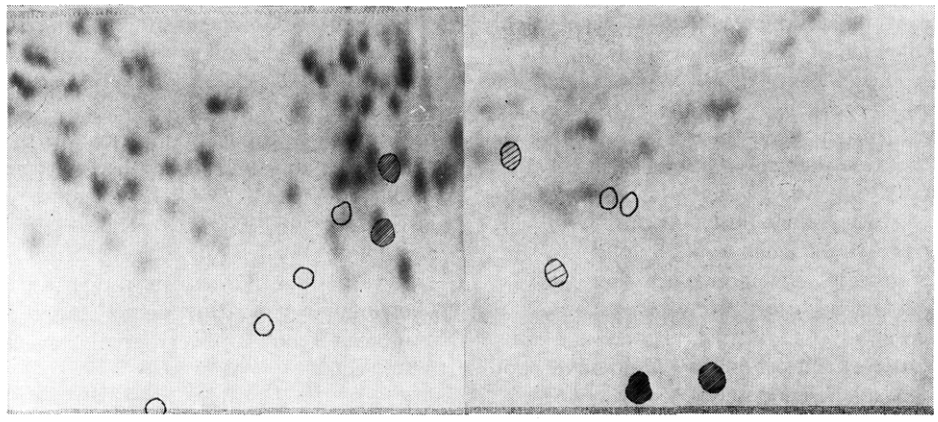

FIGURE 4: Fingerprint developed with ninhydrin of a dansyl fibrinogen preparation with the fluorescent spots drawn in. The intensity of the latter is indicated by the degree of crosshatching: origin, top center; electrophoresis in horizontal direction, negative pole to the left, positive one to the right; chromatography in vertical direction.

after the ninhydrin staining and no ninhydrin color appears in their place. Apparently the dansylated peptides are soluble in acetone and washed away during dipping into the ninhydrin solution. Careful spraying with the reagent did not affect the fluorescence and at the same time a colored spot appeared in the same place.

ACTION OF THROMBIN UPON THE LABELED FIBRINOGEN. In agreement with results already reported (Johnson and Mihalyi, 1965), the clottability of samples of low degree of labeling (6 or less) was the same as that of the parent fibrinogen. With higher labeling no firm gel was formed and with dansyl-F-27 a loose precipitate-like mass was obtained. The clottability with these preparations was slightly lower, ranging from 94% to as low as 87% in some cases. The clot, or the precipitate, was packed by centrifugation in a high-speed centrifuge and the fluorescence of the supernatants was measured. Most of the fluorescence was attached to the clot in the low-labeled conjugates, the amount remaining in solution ranging between 7 and 11% of the total fluorescence of the original material. Higher amounts were found in the supernatants of the highly labeled samples, with a value as high as 29% in one case, the usual values being 12 to 17%.

The fibrinopeptides separated from the clot liquor by chromatography on a Sephadex G-50 column were not fluorescent. All the fluorescence was present in the high molecular weight fraction eluted with the elution front. This fraction represents, therefore, the labeled, originally nonclottable proteins and fibrinogen which became unclottable by the labeling. The lack of fluorescence of the peptides confirms the finding mentioned already that the N terminal of the α chain was not labeled and also shows that the ϵ-NH_2 group in fibrinopeptide B did not react with the dye either.

Discussion

General Properties. The binding of the first 6–8 dansyl residues has a very small effect on the general properties of the fibrinogen molecule. Sedimentation, salting out, and optical rotation are practically unchanged. Clottability also remains unaffected, proving that the groups on which substitution occurred are not involved in this reaction. Further labeling, however, shows more drastic changes. The salting out curves indicate a transition, centered at about 14 bound dansyl residues, to a more easily salted out variety. The optical rotation data show a more gradual change. Apparently, the accomodation of the dansyl residues above a certain number necessitates a minor reorganization of the molecule. The change of solubility may be a reflection of this, or simply of the attachment of the bulky, hydrophobic naphthalene groups. At the same time, the clot-forming ability disappears. It is not possible to decide at this point whether the latter is caused by blocking of specific residues, or simply by steric hinderance caused by the interposition of the dansyl residues at the points of attachment of the polymerizing fibrinogen molecules.

Chemical Considerations. Some of the phenolic hydroxyl groups of the tyrosine residues of fibrinogen react more readily with dansyl chloride than its ϵ-NH_2 groups. This is especially evident under the unfavorable reaction conditions of pH 6–7. Within this pH range there are 4.7 times as many tyrosine hydroxyl groups dansylated as there are ϵ-NH_2 groups. This is in spite of the approximately twofold excess of lysine over tyrosine residues. Increase of pH equally favors the dansylation of these two groups (Gros and Labouesse, 1969). Therefore, the initial advantage of the tyrosyl residues, caused by the apparently more reactive two or three specific tyrosine residues, is gradually balanced and in the high-labeled protein there are about equal numbers of both kinds of labeled residues. The amount of dansylsulfonic acid, estimated by elution of the thin-layer chromatograms, approximately corresponds to the expected hydrolytic destruction of the dansyltyrosine residues, and it was much less than this when estimated by the direct method. Therefore, it is unlikely that other labile dansyl derivatives were formed, such as the imidazole ones, which are destroyed during hydrolysis, as shown by Hartley and Massey (1956). Equally unlikely appears the possibility of noncovalently bound dansylsulfonic acid, so tightly bound that it did not separate during the preparation of the dansyl conjugate. Nevertheless, an experiment was performed mixing native fibriongen with dansylsulfonic acid equivalent to the dansyl chloride used in the dansylation reaction and under the same reaction conditions. The mixture was passed through a Sephadex G-25 column and optical density and fluorescence of the eluates was monitored. The protein and the sulfonic acid peak were well separated. The former had a slight fluorescence, corresponding to 0.07 mole of dansylsulfonic acid per mole of fibrinogen. Thus, the presence of noncovalently bound dansylsulfonic acid can be excluded.

The number of susceptible sites in fibrinogen, *i.e.*, sum of

lysine, tyrosine, and α-NH_2 residues, is 319 (Mihalyi *et al.*, 1964). Thus, even in the highest labeled fibrinogen only 11.3% of the sites were occupied. Assuming a random labeling of these, the number of fluorescent spots on tryptic peptide maps should be equal to half the number of sites, one-half of the total number because each chain has its identical pair. It should be emphasized that when a ϵ-NH_2 group is dansylated a site susceptible to trypsin is lost and the fluorescent peptide corresponds to two adjacent tryptic peptides of the native molecule. Nevertheless, the number of these will be equal to half the number of susceptible sites, because the chance of labeling two adjacent lysine residues and thus having three of the native peptides in one fluorescent spot is only 1:10 in the highest labeled material. Contrary to these expectations, the number of fluorescent spots on the tryptic peptide maps was small, and of these only about four were constantly present and of high intensity. Therefore, one has to assume that there are a small number of specific sites on the molecule, which react more readily with dansyl chloride than the rest of the residues of the same type. Conversely, the data reported here show that two theoretically possible sites, the N-terminal glutamic acid residues of the α chain and the ϵ-NH_2 group close to the N terminus of the β chain, do not react with the label.

It is surprising that the susceptible groups on the parts of the molecule which are removed by thrombin as fibrinopeptides are not labeled, since these are pictured as relatively loose parts of the molecule. In contrast, the N-terminal tyrosine residue of the γ chain is partially labeled even under the unfavorable reaction conditions of pH 6–7 and fully reacted in the highly labeled conjugates. The dye is attached to both the α-amino and phenolic hydroxyl groups, because thin-layer chromatography did not reveal any α-dansyltyrosine and the near stoichiometric amount of didansyltyrosine in the high-labeled material shows that very little, if any of this residue was labeled only on the hydroxyl group.

The high affinity of some sites and refractoriness of others for the naphthalene dye cannot be explained at the present. All the susceptible sites, ϵ-amino groups, and phenolic hydroxyl groups, react freely with hydrogen ions (Mihalyi, 1968, 1970), and there are apparently no subclasses in either of these with respect to their titration. The large difference in size between the two reagents, hydrogen ions, or naphthalene groups, obviously make steric factors a strong contender for explaining the selectivity of the latter. With these, hydrophobic bonding may be also a factor, although, the fairly high polarity of their environment and their high degree of exposure to solvent in the conjugate (Mihalyi and Albert, 1971) indicate that this factor cannot be very important.

Although the number and nature of specific sites probably is characteristic for each individual protein, the fact that both the ϵ-amino and the tyrosine hydroxyl groups are labeled in dansylated proteins must be of more general significance. This heterogeneity in the binding sites may serve as an explanation for heterogeneity of dansylated proteins with respect to their fluorescent properties and indeed, in the following paper it will be demonstrated that with dansylated fibrinogens there is a very good correlation between these two aspects.

References

Chen, R. F. (1968a), *Arch. Biochem. Biophys. 128*, 163.

Chen, R. F. (1968b), *Anal. Biochem. 25*, 412.

Custer, G., III (1969), *J. Chromatogr. 42*, 429.

Easley, C. W. (1965), *Biochim. Biophys. Acta 107*, 386.

Gray, W. R. (1967), *Methods Enzymol. 11*, 139.

Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7*, 463.

Hartley, B. S. and Massey, V. (1956), *Biochim. Biophys. Acta 21*, 58.

Johnson, P., and Mihalyi, E. (1965), *Biochim. Biophys. Acta 102*, 476.

Jork, H. (1968), *in* Quantitative Paper and Thin-layer Chromatography, Shellard, E. J., Ed., New York, N. Y., Academic, p 79.

Laki, K. (1951), *Arch. Biochem. Biophys. 32*, 317.

Mihalyi, E. (1965), *Biochim. Biophys. Acta 102*, 487.

Mihalyi, E. (1968), *Biochemistry 7*, 208.

Mihalyi, E. (1970), *Biochemistry 9*, 804.

Mihalyi, E., and Albert, A. (1971), *Biochemistry 10*, 243.

Mihalyi, E., Small, P. A., Jr., and Cooke, J. P. (1964), *Arch. Biochem. Biophys. 106*, 229.

Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 82*, 596.

Rasmussen, P. S. (1955), *Biochim. Biophys. Acta 16*, 157.

Rinderknecht, H. (1962), *Nature (London) 193*, 167.

Weber, G. (1952), *Biochem. J. 51*, 155.

White, F. H., Jr. (1964), *J. Biol. Chem. 239*, 1032.

Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133*, 369.

Yang, J. T., and Doty, P. (1957), *J. Amer. Chem. Soc. 79*, 761.