Tanford, C., Kawahara, K., Lapanje, S., Hooker, T. M., Jr., Zarlengo, M. H., Salahuddin, A., Aune, K. C., and Takagi, T. (1967b), *J. Am. Chem. Soc. 89*, 5023.

Tanford, C., Pain, R. H., and Otchin, N. S. (1966), *J. Mol. Biol. 15*, 489.

Tanford, C., and Taggart, V. G. (1961), *J. Am. Chem. Soc. 83*, 1634.

Timasheff, S. N., Mescanti, L., Basch, J. J., and Townend, R. (1966), *J. Biol. Chem. 241*, 2496.

Timasheff, S. N., and Townend, R. (1961), *J. Am. Chem. Soc. 83*, 470.

Townend, R., Weinberger, L., and Timasheff, S. N. (1960), *J. Am. Chem. Soc. 82*, 3175.

Physicochemical Studies of Bovine Fibrinogen. IV. Ultraviolet Absorption and Its Relation to the Structure of the Molecule*

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ABSTRACT: The specific extinction of bovine fibrinogen was determined under various conditions. From the alkaline spectrum the tyrosine and tryptophan content of the molecule was calculated. The neutral and alkaline spectra are in good agreement with spectra calculated on the basis of the number of aromatic amino acid residues found. The spectrophotometric titration of the tyrosine residues was performed in the native, alkali-, urea-, and guanidine hydrochloride denatured molecule. The titration curves were analyzed with respect to electrostatic interaction and intrinsic p*K*. There was no indication of more than one class of dissociating tyrosine groups, or of groups not ionizing at all in the native molecule. Perturbation spectra showed that only about 33% of both tyrosine and tryptophan residues are in contact with the solvent. Acid and alkali denaturation raised this figure; however, it did not bring about complete exposure of the buried residues. The perturbing power of the protein fabric was estimated from the difference spectra.

The study of the absorption of ultraviolet radiation by proteins provides answers on several different levels of inquiry into the protein structure. As an analytical method it is useful in determining the amounts of aromatic amino acids, which, because of their lability, are difficult to estimate even with the present day techniques (Beaven and Holiday, 1952). Furthermore, it can be utilized to investigate the ionizing ability of the tyrosine residues and also the nature of the environment in which these and the other aromatic amino acid residues are located (Wetlaufer, 1962).

Some of the tyrosyl residues in certain proteins are unable to ionize in the native molecule, but their ionization becomes normal after disrupture of the secondary and tertiary structure of the protein. The ionization of the phenolic hydroxyl groups results in a large increase in the ultraviolet absorption; therefore, ultraviolet spectral changes, in the pH range where these groups ionize, can be used to follow the denaturation of these particular proteins.

Whereas the above method is applicable only to the alkaline denaturation of the protein, the method of difference spectroscopy, which detects small perturbances in the absorbing properties of the chromophores, is of more general usefulness. It is not restricted to the tyrosyl groups either, but is applicable to all the chromophoric residues. In the native state some of these are buried in the hydrophobic regions of the molecule and become accessible to solvent only after disruption of the secondary structure. The change in environment accompanying this process is reflected in the absorbing properties of the molecule. These changes in absorptivity are one order of magnitude smaller than the ones caused by the phenoxide ionization; nevertheless, they can be used to great advantage in the study of denaturation kinetics of the protein molecule.

The difference spectrum, besides its use as an indicator of a change in the conformation of the molecule, can provide also information on the number and nature of buried chromophores, since each of these has a characteristic perturbation spectrum. By the use of perturbants of various molecular sizes one may find that some of the chromophores are located in crevices accessible to smaller but not to larger perturbants (Herskovits and Laskowski, 1960, 1962a,b). This method can furnish, therefore, valuable data on the surface topography of the native molecule.

Schauenstein and Hochenegger (1953) have shown

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that some of the tyrosyl residues in fibrinogen have abnormal ionization characteristics. Also, it has been suggested that tyrosyl residues play a central role in the polymerization of fibrin monomers (Laki and Mihalyi, 1949; Sizer and Wagley, 1951; Mihalyi, 1954). A detailed study of the ultraviolet absorption of fibrinogen may thus reveal some of the peculiarities of these groups and eventual changes in them during the proteolytic action of thrombin upon fibrinogen, or the polymerization of the fibrin monomers.

Materials and Methods

Bovine fibrinogen (fraction I, from bovine plasma) (lots L 210, T 4105, and V 5201) from Armour Pharmaceutical Co., Kankakee, Ill, was purified either by the procedure of Laki (1951), or by that of Blombäck and Blombäck (1956). The purified protein was dialyzed exhaustively against 0.3 M KCl in the cold. Since the solutions have a tendency to become more turbid on standing at low temperature, they were always clarified immediately before use by 30-min centrifugation in the no. 30 head of the Spinco Model L preparative ultracentrifuge at 25,000 rpm. Clottability of the preparations, assayed as described by Laki (1951), ranged from 96.0 to 96.5% with the preparations according to Laki, and was better than 98% with the ones prepared according to Blombäck and Blombäck.

Thrombin was purified from Thrombin, Topical of bovine origin, from Parke Davis and Co., Detroit, Mich., by the method of Rasmussen (1955) to a specific activity of approximately 300 NIH units/mg of protein. Guanidine hydrochloride was prepared from Eastman Organic Chemicals guanidine carbonate. At 5 M concentration the optical densities of the purified substance were below 0.05 down to 250 mμ. Urea was recrystallized from 70% ethanol. The solutions, except those of fibrinogen, were clarified before use by filtration through Corning UF sintered-glass filters.

Protein concentrations were determined initially by dry weight measurements of protein solutions and their dialysates as described previously (Mihalyi *et al.*, 1964). After the optical density to protein concentration conversion factor had been established, protein concentrations were estimated by optical density measurements.

Ultraviolet absorption measurements were performed with the Beckman DU quartz spectrophotometer equipped with the photomultiplier attachment, or with the Cary Model 14 M recording spectrophotometer. The wavelength scale of these instruments was calibrated with a low-pressure mercury light, as recommended by the manufacturers, and found to be correct to better than 0.2 mμ in the 250–330-mμ range. The slide wires were calibrated by absorption measurements of standard alkaline K_2CrO_3 solutions as described by Haupt (1952), and found to be correct to 0.01 ODU over the 0–1.0-OD range. Fluorescence and stray light effects were estimated as described in a previous communication (Mihalyi, 1965a) and optical densities were always kept below the limit where these became noticeable. In the difference spectroscopy work the 0–0.1 slide wire, with the Cary spectrophotometer, greatly facilitated compliance with the above requirements. This slide wire proved to be accurate to within 0.002 ODU, when tested with a tenfold diluted alkaline standard chromate solution. However, its use placed very stringent demands on the matching of the protein concentrations in the two solvents. The most suitable method found was to deliver 1-ml aliquots of fibrinogen solution of suitable concentration from a Gilmont ultramicroburet into a 25-ml volumetric flask, which contained all the ingredients with a volume deficiency of slightly more than 1 ml, and then completing the volume accurately with distilled water. With most of the measurements the cell compartment was thermostated to 25°, while with some others the temperature was maintained at 8°.

The solutions were placed in matched Suprasil or Quarasil cells from Precision Cells, Inc., New York, of 1.000-cm light path. With the Cary spectrophotometer a base line was always recorded with both cells filled with solvent and the optical densities were corrected with respect to this. In the difference spectrum studies the base line was obtained by recording the perturbing solvent against the reference one. When the former had appreciable absorption, as with ethylene glycol, urea, or guanidine hydrochloride solutions, the tandem arrangement was used with four separate, matched cells. Some of the guanidine hydrochloride preparations, although low in optical density, contained an impurity which had an increase in absorption upon alkalinization. This necessitated the use of the tandem arrangement with the titrations in guanidine hydrochloride, in spite of the fact that guanidine was present in both cells. The sensitivity of the instrument was varied to record the difference spectra with varying slit widths and sufficient sensitivity was used to make the peak heights virtually independent of the latter.

pH measurements were performed with a Cambridge Model R pH meter, equipped with a Beckman general purpose glass electrode and a saturated KCl–calomel reference electrode, or with the Radiometer PHM 4c pH meter in connection with Leeds & Northrup miniature electrodes, catalog no. 124138. The solutions were contained in a jacketed vessel, thermostated to 25°, and a current of CO_2-free N_2 was blown over them to avoid absorption of atmospheric CO_2. The alkaline error of the glass electrode was determined by the method of Levy (1958) and was found negligible with both electrode systems in the presence of 0.3 M KCl.

The spectrophotometric titration curves were obtained by two methods. The first consisted of measuring the optical density of fibrinogen solutions of the same concentration but at various pH values against corresponding blanks in the Beckman spectrophotometer. Readings were made at four wavelengths, three around the peak of the phenoxide ion absorption, 292, 295, and 298 mμ, and one at 330 mμ. The last reading served for turbidity correction. When it was small it was subtracted from the readings. When it was appreciable, as with the alkali-denatured fibrinogen solutions below pH 10.5, the inverse fourth power of the wavelength

relationship was used to evaluate the turbidity correction. The difference between the corrected optical density of an alkaline and a neutral solution at the same wavelength was considered to be proportional to the degree of ionization of the tyrosine residues. In calculating the latter it was assumed that at pH 7 none and at pH 13 all of the tyrosine residues are in their ionized form. With this procedure separate titration curves were constructed at each one of the above-mentioned wavelengths. The second method for obtaining the titration curves, if not more accurate, was more explicit in discovering secondary effects and consisted of recording the difference spectra of alkaline solutions of fibrinogen against a neutral solution of the same protein at the same concentration. With this method the pen was zeroed at 330 mμ and the recording checked for the presence of the two isosbestic points. If turbidity was present the curve was above the base line at these points and a scattering curve was constructed graphically, which started at the base line at 330 mμ and then intersected the absorption curve at the wavelengths of the isosbestic points. The turbidity at the wave length of the peak of the difference spectrum was read on this curve and subtracted from the height of the difference peak. The corrected peak heights leveled off at about pH 13 and the maximum height attained there was considered to correspond to the complete ionization of the tyrosine residues. Intermediate heights were converted to fractions of groups in their ionized form by the use of the maximum height. The first method was used in the early stages of this work and the second later, but since the results with the two were in agreement, they will be presented without identification of the method used.

The pH of the solutions was adjusted with primary–secondary phosphate, glycine, Tris-HCl, or secondary–tertiary phosphate buffers in their respective buffering ranges. Above pH 11 buffer-free solutions were also used, where the concentration of KOH in the solution determined the pH. The ionic strength of the buffers was adjusted to the same level supplementing them with the proper amount of KCl.

Results

Specific Extinction of Neutral Fibrinogen. Fibrinogen solutions scatter light to a larger extent than smaller molecular weight proteins; therefore, a correction for scattering effects is advisable in estimating the specific extinction of this protein. Since neither of the amino acids absorbs at 320 mμ, the apparent extinction of a protein solution at this wavelength, in the absence of other chromophores, must be caused by scattering. The absorption spectra can be corrected for scattering effects as recommended by Schauenstein and Bayzer (1955); however, attempts to obtain scattering spectra of fibrinogen revealed the presence of a minute contaminant, absorbing at 405 mμ, which gave rise to wavelength exponents larger than 4. Because the wavelength exponent could not be determined, a minimal correction was adopted by simply substracting E_{320} from E_{280}. This amounts to assuming that the wavelength exponent in the scattering equation is equal to zero. Assuming an inverse fourth power relationship with the wavelength, which is the largest theoretically possible scattering correction, would have increased the correction only by a factor of 1.70. This procedure appears justified because the scattering effect at 320 mμ is small, on the order of 1.1% of the absorption at the maximum, and when larger scattering is present, it is caused by large aggregates, which have a wavelength exponent much smaller than 4.

Several dilutions were made up by weight from fibrinogen solutions whose concentration was determined by dry weight measurements and their extinctions estimated at 280 and 320 mμ, at pH 7.1 and 0.3 ionic strength, in both the Beckman and the Cary instruments. The specific extinctions corrected for turbidity, calculated at four levels of concentration, from approximately 0.3–1 mg/ml, agreed within experimental error with both instruments. Therefore, an average of all the data was calculated. The constancy of the specific extinctions at various concentrations proves the linearity of optical density with protein concentration in the range studied. The results for each of the two fibrinogen preparations are listed in Table I. There was no significant difference between the preparation according to Laki and that according to Blombäck and Blombäck.

TABLE I: Specific Extinctions of Neutral Bovine Fibrinogen Solutions.[a]

Preparation	$E_{280}^{0.1\%}$	$E_{320}^{0.1\%}$
Blombäck and Blombäck (1956)	1.527 ± 0.004	0.016 ± 0.004
Laki (1951)	1.520 ± 0.005	0.019 ± 0.006
Mean	1.523	0.017
Corrected mean ($E_{280}^{0.1\%} - E_{320}^{0.1\%}$)	1.506	

[a] Each value of E_{280} and E_{320} is the average of eight determinations, with two instruments at four concentration levels.

Once the specific extinction was estimated in neutral salt solution, the values in other solvents were established relative to this. The data, corrected for turbidity with the method just mentioned, are presented in Table II. These should be compared with the ones published by Blombäck (1958), Hörmann and Gollwitzer (1966), and others, obtained in some of the solvents used in these studies.

Tyrosine and Tryptophan Content of Fibrinogen and Its Relation to the Absorption Spectrum. In strongly alkaline solution the protein structure is destroyed to a large extent and it is assumed that the ultraviolet absorption of the chromophores becomes identical with

TABLE II: Positions and Relative Heights of the Maxima and Minima of Fibrinogen Solutions in Various Solvents.[a]

Buffer (M), pH	Maximum		Minimum	
	λ mμ	*E* (rel)	λ mμ	*E* (rel)
KCl (0.3)–phosphate (0.1), 7.0	280.0	1.000	250.0	0.370 ± 0.006
KOH (0.1), 12.8	282.5	1.063 ± 0.0003	268.5	0.897 ± 0.002
	289.5	1.057 ± 0.002		
Urea (5), 7.4	279.0	1.000 ± 0.004	249.5	0.381 ± 0.007
Guanidine hydrochloride (5), 5.8	278.5	1.007 ± 0.003	249.5	0.395 ± 0.007
Guanidine hydrochloride (5), 7.6	278.0	0.996 ± 0.000	249.5	0.379 ± 0.003

[a] Values are averages of three series of determinations, each normalized with respect to the 280-mμ peak in pH 7.0 phosphate of the same series. All values were corrected for turbidity by subtracting the extinction at 330 mμ. Data obtained in the Cary, Model 14 M recording spectrophotometer, with low-scanning speed, and the chart speed was adjusted to give one division per millimicron on the chart; slit widths, 0.1–0.2 mm.

that of the free amino acids (Beaven and Holiday, 1952). Furthermore, it is assumed that above approximately 280 mμ the only absorbing amino acids are tyrosine and tryptophan; therefore, measuring the absorption at two different wavelengths and knowing the molecular absorption coefficients of these amino acids enable the calculation of their proportion in the protein. For the purpose several wavelength pairs were recommended in the literature (Holiday, 1936; Goodwin and Morton, 1946). We have used two pairs (280 and 294 mμ, and 282 and 290 mμ) which gave virtually identical results, using the extinctions corrected for turbidity as described in the preceding section. The means of the values were 29.1 residues of tyrosine and 20.9 residues of tryptophan per 10^5 g of fibrinogen. Henschen and Blombäck (1964) obtained very similar values by the use of a spectrophotometric procedure nearly identical with ours. The figure for tyrosine is in excellent agreement with our analytical value of 29.2 (Mihalyi *et al.*, 1964); for tryptophan, on the other hand, there is no reliable independent estimate.

Knowledge of the tyrosine and tryptophan content of the molecule, supplemented with the analytical figure on the phenylalanine residues, enables the calculation of the spectrum of a mixture of the free aromatic amino acids at the same residue concentration. Accurate data on the absorption of these amino acids were determined in this laboratory at every 2 mμ, in both neutral and alkaline solution, and these were used to calculate the spectra. The closeness and range of fit of the calculated to the experimental curve provide a check on the accuracy of the aromatic amino acid content obtained by fitting the curve at only two points. Furthermore, the spectra of the free amino acids undergo various shifts when the latter are built into the protein structure, while their shapes change only very slightly. These shifts are also easily discovered when comparing the experimental and calculated spectra and their approximate magnitude estimated by shifting the two along the abscissa until the best match is obtained.

The calculated and the experimental spectra are shown in Figure 1, the latter with a minimal turbidity correction obtained by raising the base line to the level of the extinction at 320 mμ. The alkaline spectrum, shown on the right-hand side, is reproduced accurately above approximately 275 mμ, showing that our first assumption was correct and in alkali the chromophores approximate the free amino acids in their absorbing properties in this spectral range. Below 275 mμ the experimental absorption is much higher than the calculated one. The cause of this discrepancy will be discussed in the next section. An independent test on the reliability of the use of free amino acids as models in highly alkaline medium and of the analytical data obtained thereby is provided by the reconstruction of the neutral spectrum with the amino acid composition obtained from the alkaline curve. As expected, the calculated curve is situated at lower wavelengths than the experimental one, but when shifted by 3.2 mμ toward the red, as it was done on the left-hand side of Figure 1, the agreement between the two is satisfactory down to about 255 mμ. The calculated curve is slightly higher than the experimental one; however, a decrease in the tryptophan from 20.9 to 20.5 residues per 10^5 g of fibrinogen reproduces the curve almost perfectly. Whether the height of the calculated curve should be the same as that of the protein is an open question (Wetlaufer, 1962). From Table II, where the heights of the native, of partially uncoiled (in 5 M urea), and of completely uncoiled (in 5 M guanidine hydrochloride) neutral fibrinogen are compared, it would appear that the difference is very small, if any; certainly below 1%. In the calculated spectra the contribution of cystine was completely neglected. The error introduced by this is small, because of the high tyrosine and tryptophan content with respect to the disulfide groups. From the data available in the literature on the absorption of the disulfide groups, this should amount to 1.2% of the total absorption at 280 mμ and to 4.4% at 260 mμ, which is about the same as the scattering correction.

Spectral Changes Occurring upon Alkalinization of Fibrinogen. Figure 2 shows a series of spectra obtained

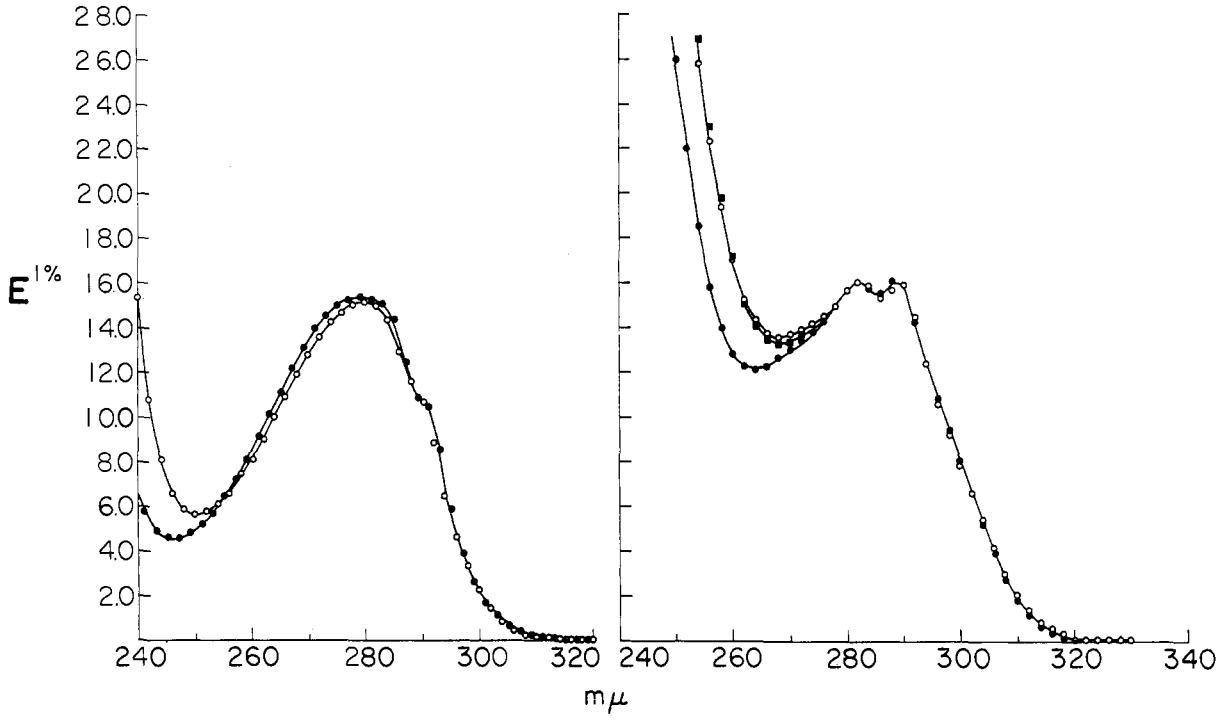

FIGURE 1: Experimental and calculated spectra of fibrinogen. Left side: neutral; right side: alkaline spectrum. Empty circles: experimental; filled circles: calculated points; filled squares: calculated points with the low-wavelength alkaline tyrosine peak shifted toward the red by 3 mμ.

at various pH values. In order to minimize time-dependent changes, each recording was completed within 3 min from the mixing of the protein solution with the respective buffer. Two well-defined isosbestic points are apparent, one at 273.5, the other at 278.0 mμ. These are situated much closer to each other than those of free tyrosine, which are approximately 10 mμ apart. The reason for this is the red shift of the absorption of the native protein against the unshifted absorption in the alkali-denatured state. Indeed, a 3.2-mμ blue shift of the neutral spectrum causes the displacement of the isosbestic points to 269 and 278 mμ, very near to their correct position, corresponding to free tyrosine. It may be mentioned here that the 278-mμ isosbestic point is insensitive to this shift because it is situated practically at the peak of the neutral spectrum, where the shift does not change the magnitude of the absorption appreciably. The behavior of the isosbestic points mentioned here indicates that the spectral changes upon alkalinization are not solely caused by the ionization of tyrosine residues. Spectral shifts should be also included in the interpretation of the changes in absorption.

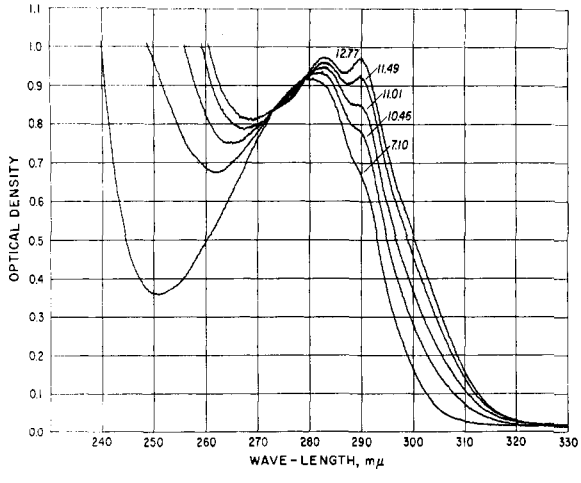

FIGURE 2: Spectra of fibrinogen at the pH values indicated on the figure, recorded immediately after buffer addition. Fibrinogen concentration 0.60 mg/ml; 1.0 slide wire.

The alkaline absorption of fibrinogen presents time-dependent changes. Repeating the recordings shown in Figure 2 after 3 hr showed a pronounced increase in apparent absorption at 330 mμ and only a blurred image of the isosbestic points. These facts point to an increase of turbidity upon standing, with a variable vertical displacement of the spectra. However, the changes in the region of true absorption were larger than expected from the increase in turbidity, suggesting that changes in the absorption of chromophores also occur during this process.

It appears from the spectra shown in Figure 2 that the large amount of tryptophan present in the molecule makes the appearance of the phenoxide ion band less

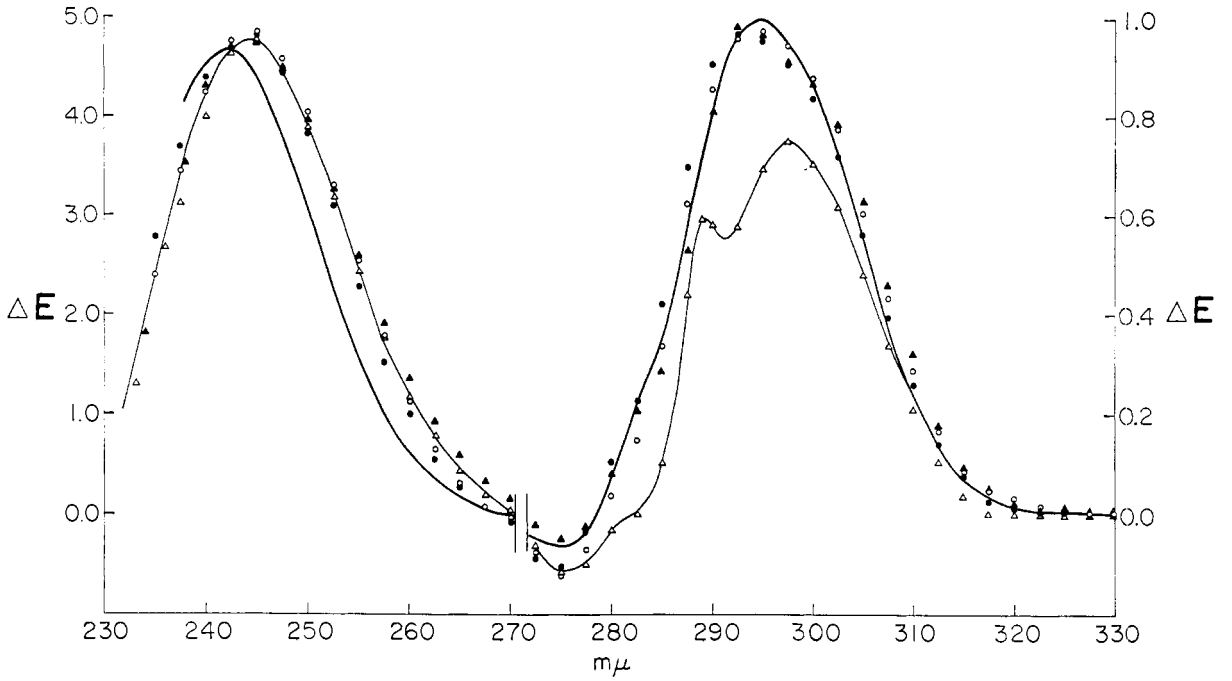

FIGURE 3: Difference spectra of fibrinogen solutions at pH intervals. The spectra were normalized to a peak height of 4.7 at the low-wavelength peak. Left-side ordinate for the low-wavelength peak, right-side ordinate for the high-wavelength peak. Empty circles: pH 10.2 native *vs.* pH 8.9 native; filled circles: pH 10.9 native *vs.* pH 10.2 native; filled triangles: pH 11 denatured *vs.* pH 11 native; empty triangles: pH 13 denatured *vs.* pH 11 denatured. Actual recordings obtained with the 0.1 slide wire at protein concentrations ranging from 0.121 to 0.637 mg/ml. Heavy line is a calculated difference spectrum of free tyrosine, pH 13 *vs.* 7, with the neutral spectrum shifted by 3.2 mμ toward the red.

conspicuous than with other proteins. This, coupled with the aforementioned facts suggesting spectral changes other than those strictly associated with the ionization of tyrosyl residues, necessitates a critical evaluation of the spectral data before they could be used to follow the titration of tyrosyl residues. For this purpose difference spectra were recorded with pairs of fibrinogen solutions at approximately the same pH interval, situated at increasingly higher pH values. With this procedure the fraction of tyrosyl groups ionizing within the range between the two pH values was isolated and contribution from other sources was consequently superimposed on a smaller background of the phenoxide absorption. This should result in a higher resolution if the tyrosine ionization and the secondary processes have different pH dependencies.

The difference spectrum of alkaline against neutral tyrosine shows two characteristic peaks, one at 242 mμ, and the other at 294 mμ, with a height ratio of 4.7. However, in the native protein the neutral spectrum is shifted toward the red. Therefore, our reference spectrum should be the difference between the alkaline spectrum of free tyrosine in its original position and the neutral one shifted toward the red by 3.2 mμ. A graphic experiment shows that this shift has little effect on the difference spectrum because of the low absorption of the neutral solution in the regions of the difference maxima. The peaks are shifted by about 1 mμ, whereas the ratio of their heights is practically unaffected. Consequently, these difference spectra are fairly insensitive to unmasking of tyrosyl residues. On the other hand, superimposed turbidity, because of its steep increase with a decrease of the wavelength, will shift slightly the position of the peaks and cause their height ratio to go up. Exposure of buried tryptophyl residues will decrease the difference peak at 294 mμ and leave the one at 242 mμ practically unaffected, resulting again in an increase in the ratio of the two. These difference spectra, therefore, provide a sensitive indicator of turbidity effects and unmasking of tryptophyl residues.

Several difference spectra obtained in the way described above are compared with the reference difference spectrum of free tyrosine in Figure 3. For easy comparison all these spectra were normalized with respect to their short-wavelength peak. The experiments with native material were run immediately after mixing with the alkaline buffer, at 8°, and the extent of denaturation during the experiment was checked by rerunning the recording in reverse direction. Up to pH 11 the two recordings did not differ appreciably, whereas above this pH the denaturation changes were of a magnitude which precluded a reliable correction for them. It appears from these recordings that up to pH 11 the shape and height ratio of the difference spectrum

of free tyrosine are accurately reproduced, but, whereas the peaks at 294 mμ coincide in their positions with that of free tyrosine, the low-wavelength peaks are shifted by about 3 mμ toward the red. It may be recalled at this point that the calculated and experimental spectra of alkaline fibrinogen, as shown in Figure 1, showed a large difference below 270 mμ which was absent in the neutral spectrum. The aforementioned difference spectra suggest that this discrepancy is caused by the shift of the low-wavelength phenoxide peak, and indeed, when this shift was incorporated in the calculated spectrum, good agreement was obtained as far down the wavelength scale as with the neutral spectrum. These results suggest that in the native molecule, up to pH 11, the spectral changes are solely caused by the ionization of tyrosine residues. The question, whether or not these groups were unmasked previous to ionization, cannot be answered because of the insensitivity of the difference spectra of tyrosine to shifts in the neutral spectrum. Certainly, unmasking of tryptophan residues could not be detected in this pH range.

Above pH 10 alkaline denaturation of fibrinogen proceeds with increasing rates and this is accompanied by, among other things, an increase in the ultraviolet absorption of the protein. To determine the cause of the absorption change, a difference spectrum was run between native and denatured fibrinogen, both being at pH 11.0. Fibrinogen in 0.15 M KCl was mixed with an equal volume of 0.1 M phosphate buffer of pH 11.0 and incubated for 10 min at 40°. This treatment ensures complete denaturation of the protein. The denatured sample was then cooled and the difference spectrum recorded at 8° against a similar mixture prepared immediately before recording. The difference spectrum obtained is included in a normalized form in Figure 3. Since the latter coincides with the difference of pairs of fibrinogen solutions at pH values not higher than 11, one may conclude that the denaturation up to this pH is accompanied by an increase of the degree of ionization of tyrosyl residues, but there is no evidence for the unmasking of tryptophyl chromophores.

An entirely different picture is obtained when the difference spectrum is recorded between two denatured solutions, one at pH 13 and the other at pH 11. This spectrum is shown also in Figure 3, normalized again with respect to the short-wavelength peak. The height of the 294-mμ peak is diminished and its shape is different from the one attributed to free tyrosine. This causes the height ratio of the two peaks to increase above the 4.7 figure. As will be shown in a later section, this effect is caused by the unmasking of some of the buried chromophores. Consequently, the denatured form at pH 11 is significantly different from the one at pH 13. The chromophores which are buried in the protein fabric in the native state are still buried at pH 11 in the denatured form, but are unmasked in the latter when the pH is raised to 13. The two denatured forms appear to be completely interconvertible, since the same difference spectrum is obtained whether the fibrinogen used in the experiment is denatured at pH 11, or it is denatured at pH 13 and returned to pH 11.

Similar experiments to the ones described above were performed with fibrinogen denatured in 5 M urea or 5 M guanidine hydrochloride. These showed only the difference spectrum of free tyrosine over their entire titration range, demonstrating that unmasking of the chromophores must have taken place in these solvents when all the tyrosines were still in their un-ionized form. These recordings are not shown in Figure 3, since they essentially conform to the curve type of free tyrosine. The purpose of Figure 3 is to present the two extreme types of difference spectra, and the transition from one to the other is conveniently expressed by the height ratio of the two difference peaks. A synopsis of the latter is presented in Table III. A ratio of 4.7 corresponds there to pure tyrosine ionization, whereas a higher ratio, if turbidity differences can be safely excluded, is indicative of unmasking of chromophores.

TABLE III: Height Ratios of the Two Difference Peaks Observed at pH Intervals along the Dissociation Range of the Phenolic Hydroxyl Groups of Fibrinogen.[a]

Solvent and Material	pH Interval	$\Delta E_{244}/\Delta E_{294}$
0.3 M KCl		
Native *vs.* native	10.3–7.3	4.85
Native *vs.* native	10.2–8.9	4.85
Native *vs.* native	10.9–10.2	4.80
Denatured *vs.* native	11.0–11.0	4.74
Denatured *vs.* native	12.7–7.1	5.11
Partially denatured *vs.* native	11.5–10.9	5.12
Denatured *vs.* denatured	12.7–11.0	5.81
5 M Urea		
Denatured *vs.* denatured	10.5–7.4	4.70
Denatured *vs.* denatured	11.4–10.5	4.69
5 M Guanidine Hydrochloride		
Denatured *vs.* denatured	10.0–7.5	4.67
Denatured *vs.* denatured	10.7–10.0	4.70

[a] The position of the peaks is somewhat variable and the wavelength given above refers only to the most frequently seen position. The heights were taken at the maxima, regardless of the position of the peaks.

The ratio of the two peak heights can be utilized to calculate the depression of the longer wavelength peak caused by unmasking. The depression will be given by the formula

$$\delta = 100\alpha\left(1 - \frac{R_0}{R}\right) \qquad (1)$$

where δ is the depression of the long-wavelength peak in per cent, α is the fraction of tyrosyl residues titrated within the pH values of the two solutions, R_0 is the

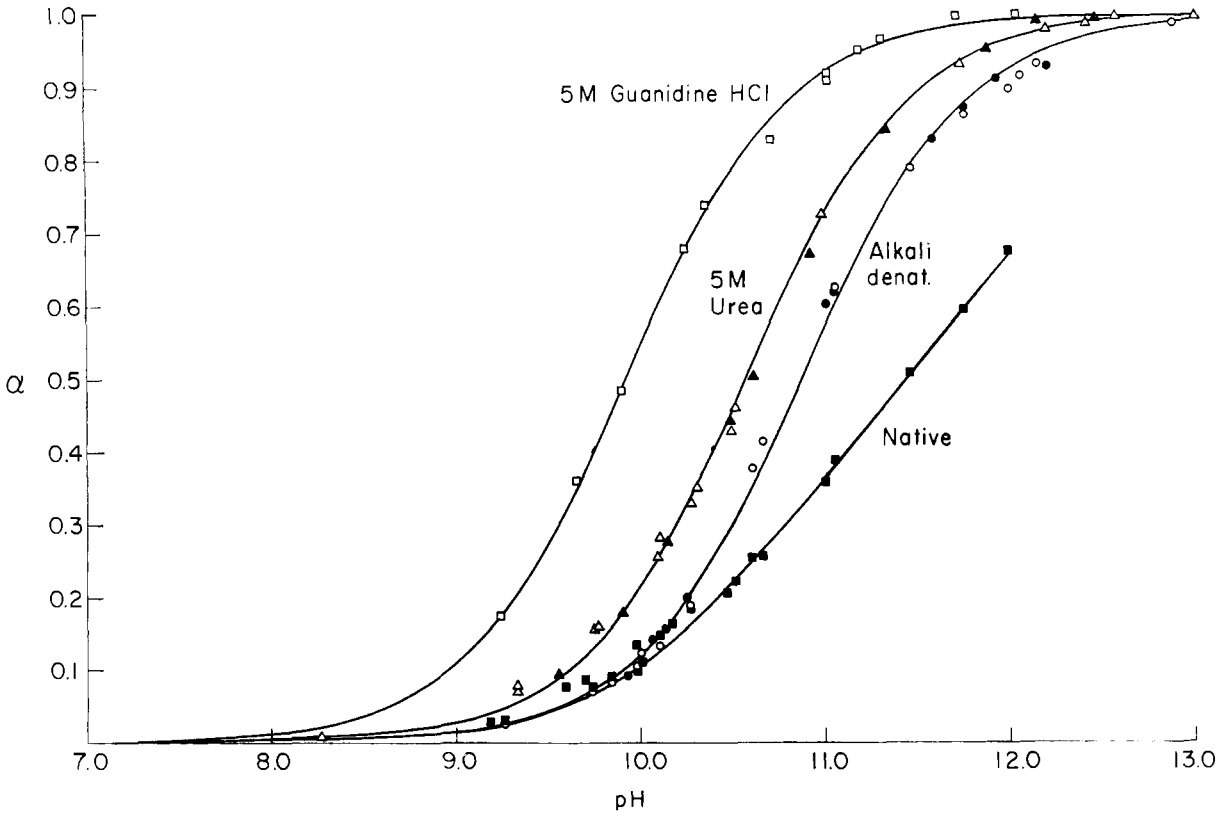

FIGURE 4: Titration curves of the tyrosyl residues of fibrinogen in various media, at 25°, and 0.25 ionic strength. Empty symbols forward, filled symbols back-titration.

normal ratio of the peaks with free tyrosine (*i.e.*, 4.7), and R is the ratio found in the experiment. This equation was derived on the assumption that the short-wavelength alkaline tyrosine difference peak is not affected by the unmasking. The ratios of the pairs of pH 12.7 denatured *vs.* pH 7.1 native, and pH 12.7 denatured *vs.* pH 11.0 denatured, 5.11 and 5.81, respectively, were used with the above formula. The value of α, taken from the titration curves which will be presented in the next section, is 1 in the first case and 0.42 in the second case. The calculations with both sets of data gave 8% depression of the height of the alkaline difference peak of fibrinogen at 294 mμ. This depression is reflected also in the number of tyrosine residues calculated from the height of this difference peak. With the known molar difference extinction coefficient of tyrosine the peak height yields 26.9 residues of tyrosine/10^5 g of fibrinogen, which is 92.4% of the expected value of 29.1. On the other hand, using the height of the 244-mμ peak gives 29.7 residues, which may be a slight overestimate caused by either small turbidity or perturbation effects. These results also provide a justification of our normalization procedure and prove that the depression observed was not produced by any arbitrariness in this procedure. The depression in the alkaline difference spectrum is the result of the difference in the number of buried groups in the alkaline and in the neutral spectrum, the latter taken as the reference. With a reference spectrum having the same number of buried groups, the height should give the correct number of tyrosine residues. The acid protein solution served this purpose with some proteins (Donovan, 1964), which are com pletely unfolded in both acid and alkali. Fibrinogen is incompletely unfolded in both these media, but it appears from optical rotation measurements (Mihalyi, 1965b) that the degree of unfolding is similar. Consequently, the unmasking effects should cancel out when the difference spectrum is recorded between an alkaline and an acid solution of fibrinogen. Indeed, under these conditions 29.1 residues of tyrosine/10^5 g of fibrinogen were calculated from the height of the 294-mμ difference peak. This result also proves that at pH 13, or near to it in 0.1 N KOH, all the tyrosine residues are in their dissociated form.

The results can be summarized in the following. In the native state the increase in optical density upon alkalinization is a true measure of the ionization of the tyrosyl residues and this is also true for alkali-denatured fibrinogen up to pH 11 at 8°. In the alkali-denatured state it appears to be a transition above pH 11, associated with unmasking of buried chromophores, and the change in absorption will reflect both the unmasking and the titration of the tyrosine residues. The titration curve, unless corrected for the unmasking, will be only approximate; however, the errors will be caused mainly by the 8% underestimate of the optical density differ-

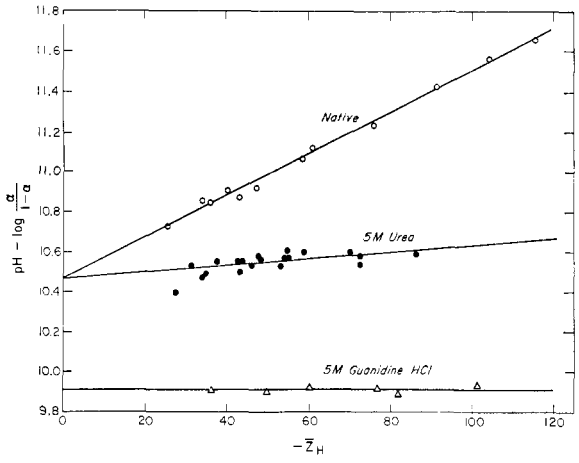

FIGURE 5: Logarithmic plots of the titration curves shown in Figure 4 (except that of the alkali-denatured fibrinogen). $\bar{Z}_H$ was calculated for an arbitrary molecular weight of 100,000, which is close to that of one spherical constituent of the molecule.

ence due to unmasking accompanied by complete ionization, and will not be very large. With fibrinogen denatured in urea or guanidine hydrochloride, these complications are no longer present and the spectrophotometric method gives the true titration curve of the tyrosine residues.

Titration Curves of the Tyrosyl Residues of Fibrinogen. The titration curves obtained in various solvents at 25° are shown in Figure 4. The curve of the native material was obtained by measurements taken 1 min after mixing with the alkaline buffer, at an ionic strength of 0.25. Subsequent measurements of the rate of denaturation showed that up to pH 11 the changes are negligible during this period. Above this pH the denaturation rate becomes appreciable; however, even at pH 11.97 the increase in ΔE_{294} is only of the order of 6% during the 1-min period. Points obtained above this pH showed a sharp upward turn, indicating that the denaturation changes are no longer negligible, and these were omitted from the graph. The samples were allowed to stand at 25° for 5 hr, after which the optical densities and the pH values were determined again. This was a sufficiently long time interval, even at pH 10.4, to assure completion of the optical density changes. In another experiment fibrinogen was denatured completely by incubation at pH 13 for 35 min, at 25°, then mixed with the various buffers. With these solutions the pH could not be lowered below 9.8 because of precipitation and the turbidity was increasing considerably below pH 10.5. The two sets of data were in close agreement, showing the reversibility of the tyrosine ionization, once fibrinogen was brought over into the denatured state. Whether or not all the tyrosines can be titrated in the native state cannot be answered directly from these data, because of the increasingly fast denaturation rate above pH 12.

Similar experiments were performed in 5 M urea, 0.25 ionic strength, and in 5 M guanidine hydrochloride. In urea forward and backward titrations were performed, starting with either neutral fibrinogen denatured in urea or alkali-denatured fibrinogen to which urea has been added. Points of both these experiments fell on the same line, demonstrating the reversibility of titration of the denatured molecule in 5 M urea.

The curves drawn on Figure 4 for alkali-, urea-, and guanidine hydrochloride denatured fibrinogen are theoretical titration curves with no electrostatic correction. The curve of the native protein has a much gentler slope than that of the previous ones. This may be caused by either electrostatic effects or heterogeneity of the titrated groups with respect to their pK's or an artifact resulting from calculating the degree of titration on the basis of the wrong assumption that all the groups present are titratable (Tanford, 1962). The last statement, perhaps, can be clarified further, using the present situation as an example. The degrees of dissociation were calculated on the basis of the extinction at pH 13, where all the tyrosines are known to be dissociated. Suppose that in the native molecule only a fraction of these can dissociate and the rest are frozen in the undissociated form. Then, at any point along the curve, the slope will be smaller than that of the theoretical titration curve comprising all the groups, by a factor equal to the titratable fraction. Obviously, in this case the titration curve should level off at the completion of the titration of this fraction, but if the denaturation becomes increasingly rapid, without special techniques, it is impossible to determine the plateau region. Schauenstein and Hochenegger (1953) used the above reasoning and calculated that in native fibrinogen only 54% of the tyrosine residues are titratable. Our data, treated similarly, yield 66% titratable groups by using the curve of alkali-denatured fibrinogen as the reference curve. The authors just mentioned used the curve of their fraction I of copper ethylenediamine treated fibrinogen for this purpose and probably this is the principal cause of the slight difference in the two estimates. However, this treatment is unacceptable because it ignores completely the electrostatic effect, which by the nature of the protein structure must be present.

A convenient way for determining the electostatic interaction factor is by plotting the titration equation according to Tanford (1962).

$$\mathrm{pH} - \log \frac{\alpha}{1-\alpha} = \mathrm{p}K_0 - 0.868w\bar{Z} \qquad (2)$$

In this equation α is the degree of titration of a certain class of groups, pK_0 is their intrinsic pK, w is the electrostatic interaction factor, and $\bar{Z}$ is the average net charge of the molecule. Plotting the left-hand side of this equation against Z should give a straight line, if w is a constant, with the intercept equal to pK_0 and the slope to $-0.868w$. Z should include the charge arising both from hydrogen ion dissociation and from binding of other ions than hydrogen. The latter is not known for fibrinogen; therefore, in this plot Z_H, the average charge derived from hydrogen ion titration alone, was

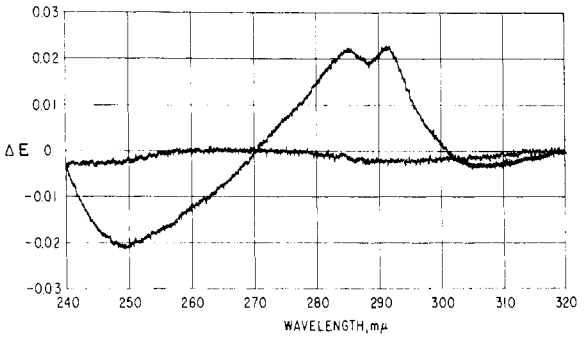

FIGURE 6: Difference spectrum of native fibrinogen in 30% ethylene glycol at pH 7.3. Reference solution in 0.3 M KCl buffer. Protein concentration 0.79 mg/ml, 0.1 slide wire, superimposed base line.

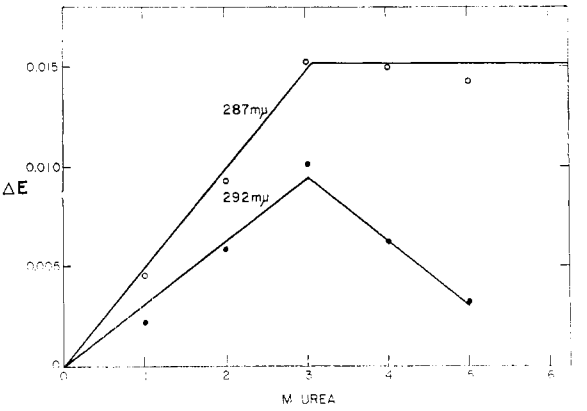

FIGURE 7: Heights of the difference spectrum peaks of native fibrinogen in increasing concentrations of urea. Recordings at 8° with the 0.1 slide wire, at 1.0 mg/ml protein concentration (pH 6.6). References solution in 0.3 M KCl buffer.

used. The hydrogen ion titration data were taken from titration curves in 0.3 M KCl and in 5 M guanidine hydrochloride. Since data for titrations in 5 M urea were not available, but titrations in 3.33 M urea up to pH 9.5 showed very little difference from those in 0.3 M KCl, the data in the latter solvent were used for urea as well. This cannot lead to substantial error because of the very slight electrostatic effect in this solvent.

Figure 5 shows the logarithmic plots according to the above equation. The linear plots obtained in the various solvents suggest that in all these cases only one single class was titrated and w was constant over the titration range. The curve of the native protein was constructed with the 1-min data and yielded 10.47 for pK_0 and 0.012 for w. When the data extrapolated to zero-time readings were used, only slightly different values were obtained for the two parameters 10.43 and 0.013. The pK_0 of the tyrosine residues in native fibrinogen is substantially higher than that of the free amino acid of 9.98. On the other hand, the values of 10.47 and 9.91 with fibrinogen in 5 M urea and 5 M guanidine hydrochloride, respectively, are very nearly equal to the pK's of 10.38 and 9.90 obtained with free tyrosine in the same solvents by the same difference spectrophotometric method. The titration of alkali-denatured fibrinogen was omitted from the figure because it appeared anomalous. The pK_0 found was 10.85, which is higher than in the native material, and w was equal to zero. The possible cause of this anomaly will be discussed later.

Estimation of the Number of Chromophores Exposed to the Solvent in the Native State. Figure 6 shows the pertubation spectrum of fibrinogen at neutral pH, caused by 30% ethylene glycol. Assuming that the conformation of the protein was not affected, the height of the perturbation peaks can be compared with that of a model mixture containing the same concentrations of the chromophoric amino acids as are present in the protein solution and from this the fraction of chromophores exposed to the solvent can be estimated (Herskovits and Laskowski, 1960, 1962a,b). When the protein contains both tyrosine and tryptophan the perturbation spectrum will be more difficult to analyze. The peak at 292 mμ will be caused almost entirely by the perturbation of the tryptophan residues, whereas both tyrosine and tryptophan will contribute to the 287-mμ peak (Donovan *et al.*, 1961; Williams *et al.*, 1965). Since the positions of their respective peaks do not coincide and may vary also from case to case, the area of the common peak could give a better measure of the perturbation than the height itself. Also, because of this, the simple arithmetic calculation of the relative proportions from the measurements of the peak heights at 287 and 292 mμ becomes approximate. Nevertheless, the tryptophan perturbation can be estimated relatively safely from the height of the 292-mμ peak and with this known, the whole perturbation spectrum can be reconstructed by trial and error. This was done by mathematical summation of the difference spectra of the two chromophores, rather than by trying to match experimentally the curve with that of a mixture of model compounds. Customarily, for these studies the ethyl esters of *N*-acetyltyrosine and tryptophan are used as model compounds. However, determinations on free amino acids and tyrosine- and tryptophan-containing peptides showed that the perturbation spectra obtained in the presence of various perturbants are within experimental error the same, whether free amino acids or peptides are used. Since at the moment the data on free amino acids are more extensive, these were predominately used in the present calculations. Although fibrinogen contains appreciable amounts of phenylalanine, the perturbation spectrum of these residues is situated at a much shorter wavelength and has a much lower intensity than those of tyrosine and tryptophan, and need not be considered here.

The curve on Figure 6 can be reproduced by the sum of the ethylene glycol perturbation spectra of free tyrosine and tryptophan. The best fit is obtained when the hypothetical mixture contains 33% of both the tyrosine and the tryptophan present in fibrinogen at the particular concentration used in this experiment.

TABLE IV: Degree of Exposure to the Solvent of the Chromophoric Residues Calculated from the Heights of the Perturbation Spectra.[a]

Material	Perturbant	ΔE_{287} Exptl	ΔE_{287} Calcd	% Exposed	ΔE_{292} Exptl	ΔE_{292} Calcd	% Exposed
Native fibrinogen	30% ethylene glycol	+0.031	+0.096	32	+0.032	+0.093	34
Native fibrinogen	3 M urea	+0.015	+0.042	34	+0.010	+0.044	22
Acid-denatured fibrinogen	30% ethylene glycol	+0.056	+0.096	58	+0.053	+0.093	57

[a] The ΔE values refer to a protein concentration of 1 mg/ml. The heights corresponding to total exposure were calculated using the perturbation spectra of free tyrosine and tryptophan, at a concentration equivalent to that in the protein present, with the respective perturbants. For these calculations the molar difference extinction coefficients of free tyrosine and tryptophan given in Table V were used.

From these figures it appears that only 33% of both the tyrosine and tryptophan residues of fibrinogen are exposed to the solvent.

A series of measurements were performed using increasing concentrations of urea as the perturbing agent. Urea, at higher concentrations, causes an instantaneous reversible expansion of the molecule, followed by a slower, irreversible denaturation (Mihalyi, 1950a,b). To avoid the latter, the experiment was performed at 8° and the recordings were done immediately after the solutions were mixed. The expansion of the reversibly denatured molecules causes a slight increase in turbidity and the recordings were corrected for this. The corrected heights are plotted against urea concentration in Figure 7. Up to 3 M urea concentration the heights increase proportionately with the urea concentration and correspond to approximately 34% of the tyrosine and 22% of the tryptophan residues being exposed to the solvent. The curve of ΔE_{287} levels off, that of ΔE_{292} decreases at higher urea concentrations than 3 M. This effect is most likely caused by a partial opening of the molecule, which exposes a few chromophores. A corresponding transition was observed on the $[\alpha]_D$ *vs.* urea concentration curve, amounting to a 7° change in the specific rotation at the D line (Mihalyi, 1965b). It would appear from Figure 7 that the tryptophan residues are involved in this transition to a larger extent and perhaps at lower urea concentrations, hence the declining side of the ΔE_{292} curve and a lower than expected percentage of the number of exposed tryptophan residues. A summary of the results of the estimation of chromophores exposed to the solvent in native fibrinogen is presented in Table IV.

Partial Exposure of Buried Groups during Acid and Alkali Denaturation. Perturbing Power of the Protein Fabric. A measure of the perturbing power of the protein fabric is the difference spectrum between the native and the fully uncoiled protein. Since the isolated chains of fibrinogen are insoluble in water, as a first approximation, a mixture of the chromophoric amino acids in a concentration corresponding to the one in the protein can be used as a model of the fully uncoiled protein. This amounts to calculating the difference between the two spectra on the left side of Figure 1, but without shifting the spectrum of the free amino acids. The above procedure assumes that the spectra of the chromophores are not shifted when they are incorporated in the polypeptide chain. The calculated difference spectrum for a protein concentration of 1 mg/ml is shown in Figure 8.

Direct recording of a difference spectrum of complete unfolding is technically not feasible; therefore, recourse was made to an indirect method suggested by Donovan (1964). Fibrinogen is partially unfolded in acid, with transfer of some of the buried groups to the surface. The number of groups involved in this process can be found by subtracting from the total number of groups exposed in the acid-denatured protein those which were already exposed in the native material. The exposure of this fraction of the buried groups produces the acid difference spectrum of the protein. Knowing the difference spectrum of the partial exposure and the number of groups responsible for it enable the calculation of the spectrum corresponding to total exposure. The estimation of groups exposed in native fibrinogen was already described and the same method was applied to the acid-denatured protein. The perturbation spectrum caused by 30% ethylene glycol at pH 1.3 was recorded. It was very similar to the one shown in Figure 6, only the peaks were higher at the same protein concentration and their height ratio slightly different to the one of the native protein. A procedure similar to the one outlined with the experiment with native protein was used to analyze the perturbation spectrum. The result of these calculations showed that in the acid solution 60% of the tyrosine and 55% of the tryptophan residues are exposed to the solvent. The corresponding figures in Table IV were obtained simply by comparing the heights of the experimental difference peaks of acid fibrinogen, perturbed by ethylene glycol, with the heights calculated for exposure of all the chromophores to ethylene glycol. These are probably less accurate than the ones obtained by curve fitting; however, the average exposure of 58% calculated there is not too far from the average of the values given above. The difference between the fraction exposed in acid-denatured fibrinogen and the fraction exposed in the native protein corresponds to the percentage of the

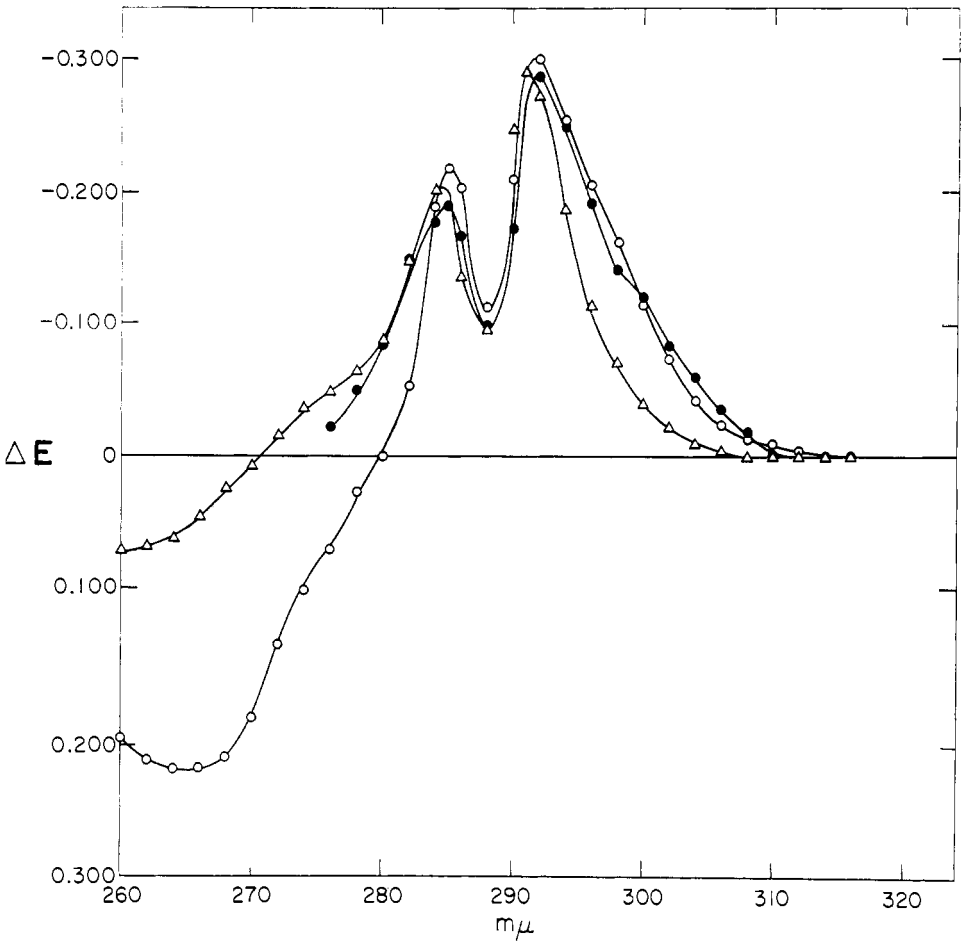

FIGURE 8: Calculated difference spectra of total and partial unmasking of the buried chromophores. Empty circles: difference between the experimental spectrum of neutral fibrinogen at 1-mg/ml concentration and the spectrum of a corresponding mixture of amino acids. Triangles: calculated difference spectrum caused by 14.2 M guanidine hydrochloride on a mixture containing 50% of the tyrosine and 66% of the tryptophan content of fibrinogen at 1-mg/ml concentration. Filled circles: difference spectrum of partial unmasking by alkali denaturation, calculated from the curves of Figure 3, for 1-mg/ml protein concentration and multiplied by 6.3 to match the curves of the total unmasking.

groups transferred from the interior to the surface during the acid denaturation of the molecule. This calculation gives 28% of the total tyrosine and 22% of the total tryptophan residues liberated during this process. The transfer of this fraction of the chromophores causes the difference spectrum shown in Figure 9. This was obtained between a solution of fibrinogen dissolved in 0.05 N HCl and one in 0.3 M KCl at pH 7.6. The peak heights of this spectrum were converted to heights corresponding to total exposure by multiplying them with the ratio of the per cent of buried groups in the native form to the per cent exposed by the acid. The result of this is 0.218 for ΔE_{287} and 0.261 for ΔE_{292} (Table VI). This is in good agreement with 0.218 and 0.298, the figures calculated from the difference between the spectrum of native, neutral fibrinogen and a corresponding mixture of the chromophoric amino acids. Also, this finding suggests that the shift in the spectrum of the chromophores, when they are incorporated into a completely unfolded polypeptide chain, must be small if any.

A reference was made already to the unmasking of buried groups in denatured fibrinogen above pH 11. This process results in spectral changes which can be best expressed as the difference between the two curves on the right side of Figure 3. In this figure, the calculated difference absorption curve of the tyrosine residues which ionize between pH 11 and 13 is shown by the heavy line. The experimental curve, drawn in thin line, is below the expected one because the perturbation spectrum of some residues exposed during this process is subtracted from the contribution of the phenoxide ions. The curves on Figure 3 are drawn on an arbitrary scale and their difference, normalized to fit the calculated curve of the total exposure of the buried chromophores, is plotted in Figure 8. The two curves have nearly identical

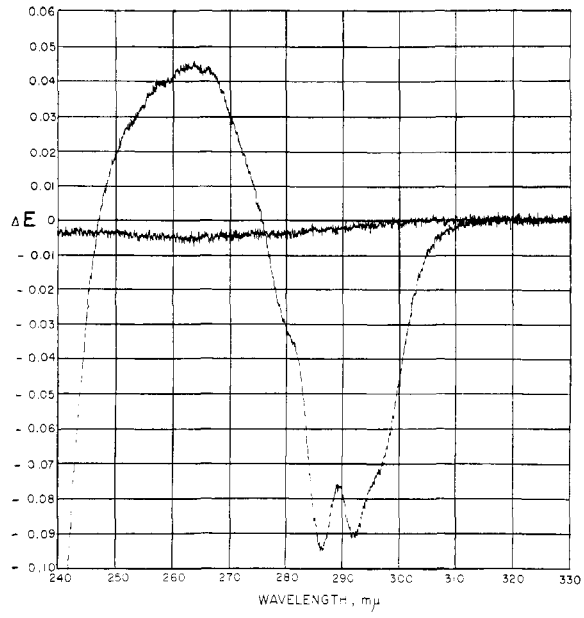

FIGURE 9: Acid difference spectrum of fibrinogen: pH 1.4 *vs.* 7.6, 0.06 ionic strength in the acid solution, 0.3 in the neutral one, 1-mg/ml fibrinogen concentration, 0.1 slide wire, superimposed base line.

shapes, showing that the difference between the expected and the experimental difference curve at high pH is indeed caused by the exposure of some of the buried residues. However, the actual heights of these double-difference peaks, expressed for 1-mg/ml concentration, are only 13–15% of those corresponding to total exposure. These figures, in view of their derivation, cannot be very accurate; also, smaller differences could have been overlooked at the lower pH intervals. At any rate, it seems that in the alkali-denatured fibrinogen at pH 13 no more than 20% of the buried chromophores are liberated.

The results of the calculations of the peak heights of the difference spectrum corresponding to the complete transfer of the buried chromophores into the solvent are summarized in Table VI. This table also contains the data pertaining to the partial exposure of the buried groups which occur during acid or alkali denaturation. An attempt was made to reconstruct the difference curve of the total exposure of the chromaphores by a calculated curve of a mixture of tyrosine and tryptophan in water against the same mixture in 14 M guanidine hydrochloride (*i.e.*, solid guanidine hydrochloride). The data obtained from the perturbation spectra of free tyrosine and tryptophan in 8 M guanidine hydrochloride were used for these computations, assuming linearity of the difference extinction up to 14 M guanidine hydrochloride concentration. Under these conditions the curve of the total unfolding was reproduced best with 50% of the tyrosine and 66% of the tryptophan residues which are present in fibrinogen. Since approximately the same fraction of these residues was found to be buried in native fibrinogen, it appears that 14 M guanidine

TABLE V: Molar Difference Extinction Coefficients of Free Tyrosine and Tryptophan in the Presence of Some Perturbants at the Peaks of Their Difference Spectrum.[a]

Perturbant	Tyrosine		Tryptophan	
	ΔE_{278}	ΔE_{285}	ΔE_{284}	ΔE_{292}
8 M urea	109	202	258	593
5 M guanidine hydrochloride	89	166	359	705
50% ethylene glycol (9 M)	157	288	425	754

[a] The difference spectra were run at approximately pH 7 and at 25°. Up to the concentrations given above, which were the highest ones investigated, ΔE increased linearily with the concentration of the perturbant.

hydrochloride is not too bad a model for the perturbing power of the protein fabric. The calculated curve is shown on Figure 8 and it appears that, except for the contribution of those groups which had an excessive red shift, the calculated difference spectrum of the total exposure of the chromophores reproduces fairly well.

Discussion

The persistence of the red shift of the shorter wavelength tyrosine peak in alkaline fibrinogen may indicate that these residues are not fully normalized. However, the longer wavelength peak appears normal; therefore, the spectral data used fot the tyrosine and tryptophan estimation are correct as far as the contribution of this amino acid goes. There is less certainty, perhaps, about the contribution of the tryptophan residues, since the latter according to some of the data presented may be partially buried even in the alkali-denatured state of fibrinogen. If this is true, the formulas should give an overestimate of the tyrosine and an underestimate of the tryptophan residues. This should be much more so with the 280–294-mμ extinction pair than with the 282–290-mμ one. Actually, the tyrosine value was in excellent agreement with the analytical one, and the two wavelength pairs gave virtually identical results. A 3-mμ shift of the absorption of about one-half of the tryptophan residues would be very conspicuous in the spectrum. However, there is no indication of this in the experimental curve, which can be reproduced very accurately with the sum of the absorption of the chromophores in their original position. Perhaps, the rather indirect evidence of only partial unmasking of the tryptophan residues in alkali should not be taken too seriously. Independent estimates of the tryptophan content of fibrinogen certainly will help to settle this point.

The titration curve of the tyrosyl residues in native fibrinogen reveals a higher intrinsic pK than expected for these groups in a protein. The possible causes of such

TABLE VI: Heights of the Difference Peaks Corresponding to Total Exposure and to Partial Exposure on Acid and Alkali Denaturation.[a]

	ΔE_{287}	Groups Transferred into Solvent (% of total)	ΔE_{292}	Groups Transferred into Solvent (% of total)
	Total Exposure			
Calculated from:				
a. Difference of experimental and calculated neutral spectrum	−0.218	68	−0.298	66
b. Difference spectra in acid, and in ethylene glycol (neutral and acid)	−0.218	68	−0.261	66
Mean Values	−0.218		−0.279	
	Partial Exposure			
Acid denaturation	−0.090	28	−0.087	21
Alkali denaturation	−0.033	10	−0.054	13

[a] Values expressed for 1-mg/ml protein concentration. See text for calculation of ΔE for total exposure. Per cent of groups transferred by total exposure is equal to the per cent of groups buried in the native state and was taken from Table IV. Per cent transferred by partial exposure is given by the ratio $\Delta E_{partial}/\Delta E_{total}$ times per cent of groups buried in the native state.

an anomalous behavior were thoroughly discussed by Tanford (1962). The value of the electrostatic interaction factor in the native molecule, on the other hand, is reasonable in view of the recent model of the molecule (Hall and Slayter, 1959). Fibrinogen, according to this, consists of three compact, spherical or nearly spherical constituents of 50–65-Å diameter, held together by loose connecting segments. The latter make up about 15% of the mass of the molecule (Mihalyi and Godfrey, 1963). The spheres are at a large distance from each other (240 Å), compared to their radius. Therefore, from the point of view of the electrostatic interaction, fibrinogen can be approximated by a globular model of 65-Å diameter. The electrostatic interaction between any two of the connected spheres is small enough and the relative proportion of the groups titrated in the intermediate sections is equally small to justify this assumption. At the ionic strength and temperature of the titration an isolated sphere of these dimensions would have a w equal to 0.020, whereas experimentally a value of 0.013 was found. The latter is entirely reasonable, since a deviation from the spherical shape or an increase by 28% of the radius can bring the two values in agreement. Expansion of the molecule at pH values higher than 10 was indeed demonstrated by Fitzgerald *et al.* (1957) and Hartley and Waugh (1960).

The value of w remains constant in the native molecule over the titration range of the phenolic hydroxyls, as far as this can be estimated without interference from denaturation. The native molecule thus appears to maintain the same conformation while the tyrosine residues dissociate. This is also evident in the optical rotation data, which show a very small change between pH 6 and 8 and then remain constant up to approximately pH 12 (Mihalyi, 1965b). The native configuration, however, is very unstable at the high pH values, as attested by the increasingly fast denaturation rate with increase of the pH. When the protein is converted into its alkali-denatured form, the electrostatic interaction term vanishes. This result can be explained, on the one hand, by a reduction of the electrostatic interaction caused by penetration of the solvent into the expanded molecule (Tanford, 1955), and on the other hand, by a flexibility of the latter, so that increasing charge causes increasing expansion, maintaining thereby the electrostatic interaction at a constant level. The zero slope of the logarithmic plot, therefore, may mean only that the term $w\bar{Z}$ is constant in eq 2, and not that w is equal to zero. Then pK_0 found from this plot is not the intrinsic pK of these groups, but the apparent value corresponding to the constant electrostatic term. This explains why pK_0 found in alkali-denatured fibrinogen is higher than the presumably true pK_0 value in the native form. A similar situation was noted with alkaline serum albumin (Tanford *et al.*, 1955a; Tanford and Roberts, 1952) and β-lactoglobulin (Tanford and Swanson, 1957).

Unfortunately, the rapid rate of denaturation made it impossible to follow, with the available techniques, the complete titration of the tyrosine residues in the native state. However, up to about 70% of their titration the groups behaved normally and there was no indication of more that one class, or inability of some of these to ionize at all, in the native protein. In contrast with this, the perturbation spectra showed that only 33% of the residues are exposed to the solvent. The two findings are, therefore, in apparent contradiction.

The perturbation spectra are very likely unable to differentiate between a situation where part of the

residues are exposed, part are buried, and the one where all are only partially exposed. Perhaps the inconsistency mentioned above could be resolved along these lines, assuming that all the groups are similar and only partially exposed to the solvent. There are no data on how far the ionization of a phenolic hydroxyl group may be affected by sinking the benzyl ring into a hydrophobic medium, while the hydroxyl groups are in contact with the solvent, but, since the phenoxide ion has a pronounced quinoid character resulting in a polarization of the benzene ring, the uncharged form will be stabilized by the nonpolar medium, and this may be the explanation of the higher than normal pK of all the tyrosyl residues. Another possibility would be a conformational change above neutral pH, but below the ionization range of the tyrosyl residues, which would completely expose the latter. This is, however, unlikely in view of the small optical rotation change seen in this region.

Similar findings were reported with serum albumins, where all the tyrosine residues are reversibly titrated, with a moderately high pK (Tanford and Roberts, 1952), and most of them appear to be inaccessible to solvent (Herskovits and Laskowski, 1962a). From the published difference spectra of lysozyme (Williams *et al.*, 1965) and α-lactalbumin (Kronman and Holmes, 1965), although they were not analyzed by the authors in this respect, it would seem that most of the tyrosine residues are also buried in these proteins. Nevertheless, their titration curves (Tanford and Wagner, 1954; Robbins *et al.*, 1967) show only one class of reversibly dissociating tyrosyl residues. However, other situations are undoubtedly present in protein molecules, since in native ribonuclease half of the tyrosine residues do not titrate at all (Shugar, 1952; Tanford *et al.*, 1955b) and also are inaccessible to solvent (Herskovits and Laskowski, 1960). This shows that the tyrosyls might be completely buried in some proteins and only partially in others, and, of course, there is no reason to believe that both these forms cannot occur simultaneously in the same protein. Distinction between partially and completely buried residues can be made also by studies of the availability of these residues to chemical modifications, along the lines suggested by Williams and Laskowski (1965).

There is a fairly complete set of data available on the unfolding of fibrinogen under various conditions inferred from optical rotation measurements (Mihalyi, 1965b). The changes in the degree of exposure of the chromophoric residues were determined under similar circumstances in the present work. A comparison of the two sets of data seems profitable. There is some correlation between changes in the number of exposed groups and the changes in helical content of the molecule. This obviously does not imply that the buried groups are within the helical regions of the molecule, but only that the disruption of the molecule seems to affect to about the same degree the various aspects of the protein structure. It is surprising in this respect the tenacity of some of the structure, as revealed by both these approaches, in the various denatured states of the molecule.

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References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Blombäck, B. (1958), *Arkiv Kemi 12*, 99.
Blombäck, B., and Blombäck, M. (1956), *Arkiv Kemi 10*, 415.
Donovan, J. W. (1964), *Biochemistry 3*, 67.
Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 2686.
Fitzgerald, J. E., Schneider, N. S., and Waugh, D. F. (1957), *J. Am. Chem. Soc. 79*, 601.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Hall, C. E., and Slayter, H. S. (1959), *J. Biophys. Biochem. Cytol. 5*, 11.
Hartley, R. W., Jr., and Waugh, D. F. (1960), *J. Am. Chem. Soc. 82*, 978.
Haupt, G. W. (1952), *J. Res. Natl. Bur. Std. 48*, 414.
Henschen, A., and Blombäck, B. (1964), *Arkiv Kemi 22*, 347.
Herskovits, T. T., and Laskowski, M., Jr. (1960), *J. Biol. Chem. 235*, PC56.
Herskovits, T. T., and Laskowski, M., Jr. (1962a), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Laskowski, M., Jr. (1962b), *J. Biol. Chem. 237*, 3418.
Holiday, E. R. (1936), *Biochem. J. 30*, 1795.
Hörmann, H., and Gollwitzer, R. (1966), *Z. Physiol. Chem. 346*, 21.
Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 526.
Laki, K. (1951), *Arch. Biochem. Biophys. 32*, 317.
Laki, K., and Mihalyi, E. (1949), *Nature 163*, 66.
Levy, M. (1958), *Compt. Rend. Trav. Lab. Carlsberg 30*, 291.
Mihalyi, E. (1950a), *Acta Chem. Scand. 4*, 317.
Mihalyi, E. (1950b), *Acta Chem. Scand. 4*, 334.
Mihalyi, E. (1954), *J. Biol. Chem. 209*, 733.
Mihalyi, E. (1965a), *Arch. Biochem. Biophys. 110*, 325.
Mihalyi, E. (1965b), *Biochim. Biophys. Acta 102*, 487.
Mihalyi, E., and Godfrey, J. E. (1963), *Biochim. Biophys. Acta 67*, 73.
Mihalyi, E., Small, P. A., Jr., and Cooke, J. P. (1964), *Arch. Biochem. Biophys. 106*, 229.
Rasmussen, P. S. (1955), *Biochim. Biophys. Acta 16*, 157.
Robbins, F. M., Andreotti, R. E., Holmes, L. G., and Kronman, M. J. (1967), *Biochim. Biophys. Acta 133*, 33.
Schauenstein, E., and Bayzer, H. (1955), *J. Polymer Sci. 16*, 45.
Schauenstein, E., and Hochenegger, M. (1953), *Z. Naturforsch. 8b*, 473.
Shugar, D. (1952), *Biochem. J. 52*, 142.
Sizer, I. W., and Wagley, P. F. (1951), *J. Biol. Chem.*

192, 213.
Tanford, C. (1955), *J. Phys. Chem. 59*, 788.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Tanford, C., Hauenstein, J. D., and Rands, D. G. (1955b), *J. Am. Chem. Soc. 77*, 6409.
Tanford, C., and Roberts, G. L., Jr. (1952), *J. Am. Chem. Soc. 74*, 2509.
Tanford, C., and Swanson, S. A. (1957), *J. Am. Chem. Soc. 79*, 3297.
Tanford, C., Swanson, S. A., and Shore, W. S. (1955a), *J. Am. Chem. Soc. 77*, 6414.
Tanford, C., and Wagner, M. L. (1954), *J. Am. Chem. Soc. 76*, 3331.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.
Williams, E. J., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3580.

Cooperative Interactions and Determination of Protein Association–Dissociation Equilibria. Hemerythrin*

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ABSTRACT: Cooperative interactions between different sites in a protein, such as hemerythrin, composed of several subunits, can be accounted for if (1) the oligomer is in equilibrium with monomer; (2) the monomer has a greater affinity for a particular ligand than does the oligomer; and (3) the monomer has a different reactivity for a given reagent than does oligomer. Further evidence for the validity of the second assumption in regard to hemerythrin has now been obtained in studies of the binding of ligands to the iron site. The apparent affinity of the protein for thiocyanate ion increases with decreasing protein concentration, as would be expected if the monomer binds more strongly. In a complementary manner, the variation of ligand binding with protein concentration may be used to evaluate the equilibrium constant K_P for $A_n = nA$. Equations and suitable extrapolations have been developed for this ligand binding analysis of dissociation, and dissociation constants and related parameters have been evaluated for hemerythrin.

Hemerythrin, a nonheme iron-containing protein of sipunculids, is composed of eight subunits (Keresztes-Nagy and Klotz, 1963a). This protein reacts with iron-coordinating ligands (Keresztes-Nagy and Klotz, 1965) and with sulfhydryl reagents (Keresztes-Nagy and Klotz, 1963b). These reactions are easily and independently detectable. When an external ligand is bound by the iron the visible spectrum of the protein is changed; in contrast, reaction with sulfhydryl reagents results in dissociation of the octameric protein to monomers. On the other hand, these two reactions are not fully independent of each other. When the protein iron is not coordinated with external ligands the SH groups react only slowly with organic mercurials or *N*-ethylmaleimide; in the presence of iron-coordinating anions, such as azide or thiocyanate, the SH groups react readily (Keresztes-Nagy and Klotz, 1965).

The dependence of the SH group reactivity on the environment about the iron is an example of cooperative interactions. A reaction at one site of the protein affects the reactivity of a second site. A molecular interpretation of this phenomenon in hemerythrin has been proposed (Keresztes-Nagy and Klotz, 1965). The model described is based on three assumptions. (1) The octameric protein is in equilibrium with a small amount of monomer; (2) the monomer has a greater affinity for iron-coordinating anions than does the octamer; and (3) the SH group in the monomer is more reactive than the SH group in the octamer. This model may be represented schematically as

$$A_8 \rightleftharpoons 8A$$

$$A + B \longrightarrow A\text{–}B$$

where A_8 is the octamer, A the monomer, and B the sulfhydryl reagent. The assumptions of this model are sufficient to explain the cooperative interaction; the attachment of an anion such as thiocyanate or chloride to iron of the protein shifts the protein dissociation

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