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Hydrogen Ion Titration Curves of the Native Bovine Fibrinogen and of Bovine Fibrinogen Denature of 5 M Guanidine Hydrochloride*

Elemer Mihalyi

ABSTRACT: Titration curves of bovine fibrinogen were obtained at four ionic strengths, at 25°, and at three temperatures at 0.3 ionic strength and also in the presence of 2.02–8.08 M formaldehyde and in 5 M guanidine hydrochloride. Measurements performed in salt solutions, below the isoelectric point, pertain to the denatured molecule, those above this point to the native molecule. Titrations in guanidine were on the denatured molecule. The following stoichiometry gave a good fit to all the data: 119.0 carboxyl (α-, γ-, δ-, and sialic acid carboxyls combined), 15.5 imidazole, 1.2 α-amino, 63.0 ϵ-amino, 29.2 tyrosyl, and 45.0 arginine residues per 10^5 g of fibrinogen. These are in very good agreement with the analytical values. The intrinsic pK's were within their normal range, except that of the guanidino groups, which was low in the native molecule and was normalized in 5 M guanidine hydrochloride. The electrostatic interaction term was smaller in the acid range than in the alkaline one, suggesting an expansion of the molecule on acid denaturation. The isoionic point was found at pH 6.6, more than one unit above the assumed isoelectric point. This suggests the binding of approximately eleven chloride ions to the isoionic protein.

The alkaline section of the titration curves of the native protein can be fitted equally well with large variations in the values of the assumed parameters. The ambiguity was resolved only by the use of all the data from the various types of titrations mentioned above. Simulated titration curves were computed to show the effect of heterogeneity with respect to pK, within one group, on the apparent pK and electrostatic interaction term.

Hydrogen ion titration curves of fibrinogen have been reported by several authors (Nordbö, 1927; Chaudhuri, 1948; Shulman and Ferry, 1950; Mihalyi, 1954). These studies were performed mainly in conjunction with various investigations of the clotting of this protein; therefore, they were restricted to a narrow pH range around neutrality where clotting occurs. Besides the function-oriented motivation of a limited range, an extension of the titration to extreme pH values seemed unprofitable in the absence of adequate knowledge of the behavior of this protein under these conditions. With recent data on the stability and the structural changes of fibrinogen in acid and alkali (Mihalyi, 1965), an interpretation of a complete titration curve was made possible. Therefore, a detailed study of this problem was undertaken and the data obtained are presented in this paper. Titrations were performed at various ionic strengths and temperatures and in the presence of formaldehyde. These were analyzed for the stoichiometry of the various titrated groups and their intrinsic dissociation constants and for electrostatic effects. Titrations were obtained also in the presence of 5 M guanidine hydrochloride. In the latter solvent the protein should approximate a random polypeptide chain, essentially devoid of all the electrostatic and other complicating factors brought about

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by the secondary and tertiary structure in the native state. Therefore, the analysis of the curves in guanidine hydrochloride should yield more reliable data on the number of groups titrated in each of the various classes of titratable groups.

Materials and Methods

Fibrinogen was purified from bovine fibrinogen (fraction I, from bovine plasma), lots T4105 and V5201, from Armour Pharmaceutical Co., Kankakee, Ill., by Laki's method (Laki, 1951). The clottability of the purified material, tested with the procedure of the same author, ranged from 95 to 96%. The solutions were dialyzed against four changes of large volumes of 0.3 M KCl in the cold. The last dialysate did not react with Nessler's reagent. The fibrinogen solutions were clarified by centrifugation in the Sorvall type SS-1 centrifuge and their protein concentration was estimated by optical density measurements at 280 and 320 mμ in the Cary Model 14 recording spectrophotometer, as described previously (Mihalyi, 1968). For the specific extinction at 280 mμ, corrected for turbidity, an $E_{cm}^{0.1\%}$ value of 1.506 was used.

Guanidine hydrochloride was prepared from guanidine carbonate, (Eastman Organic Chemicals), following the procedure of Nozaki and Tanford (1967a).

A commercial 40% formaldehyde solution of reagent grade was used which contained 12–15% methanol, added by the manufacturer. Therefore, the titration mixtures also contained a small amount of methanol, varying from 10% with the highest to 1.25% with the lowest formaldehyde concentration used. Before use, the solutions were either adjusted to pH 7, or passed through a mixed-bed resin column; the pH readings of the latter solutions were 5.1–5.7.

Mixed-bed resin (AG501-8X) was obtained from Bio-Rad Laboratories, Richmond, Calif. All the other reagents were reagent grade commercial preparations.

pH Measurements. A Radiometer Model PHM 4c pH meter was used with either of two electrode systems: (1) Beckman general-purpose glass electrode (cat. no. 41252) and sleeve-junction, calomel reference electrode (cat. no. 40463), and (2) Leeds & Northrup, miniature pH electrode (cat. no. 124138). The titration vessel was thermostated to ±0.05° of the desired temperature. The electrodes were standardized with 0.05 M potassium biphthalate to which a pH of 4.000 was assigned at 25° and also by titration of the solvent with standard acid or alkali, as will be described for the determination of apparent activity coefficients. Standardization was repeated at the end of each series of measurements.

For the measurement of the pH of salt-free solutions the jacketed vessel with the glass electrode was connected by a narrow bore tube to a second vessel with the reference electrode. In this way the contamination of the solution around the glass electrode by salt leaking out of the reference electrode was avoided. These measurements were performed with a Cary Model 31 vibrating reed electrometer.

Conductivities were determined with a Radiometer Model CDM 2d conductimeter, equipped with a type CDC 114 conductivity cell.

Titration Procedure. Samples (20 ml) of solvents, or approximately 1% protein solutions, were titrated with acid or alkali which had been adjusted with KCl to the same ionic strength as that of the sample, and added from a 1-ml Gilmont ultramicroburet. In the titrations with alkali the vessel was closed and argon saturated with water vapor was blown over the surface of the mixture. Individual samples were prepared in the range of precipitation of fibrinogen, and also above pH 10, where time-dependent changes were observed. In the first case the mixtures were allowed to equilibrate overnight in the cold, and in the second, measurements were taken 2 min after the alkali addition. Continuous titrations were used in the pH range of solubility and stability of fibrinogen. These were always checked by a few points obtained on individual mixtures. No difference was found between the results of the two methods.

The effect of temperature was investigated on individual mixtures titrated to various degrees. Water from three thermostated water baths, each at a different temperature, could be circulated through the electrode vessel's jacket.

TABLE I: Apparent Hydrogen and Hydroxyl Ion Activity Coefficients and pK of the Guanidinium Ion.

Solvent	Temp (°C)	$-\log \gamma_{H^+}$	$-\log (K_W/\gamma_{OH^-})$
0.05 M KCl	25.0	0.070	13.776
0.15 M KCl	25.0	0.077	13.772
0.30 M KCl	25.0	0.069	13.772
0.60 M KCl	25.0	0.052	13.766
0.30 M KCl	9.55	0.053	14.284
0.30 M KCl	38.0	0.067	13.372
			pK (Gd)
5 M Gd·HCl (Leeds & Northrup electrode)	25.0	−0.611	13.575
5 M Gd·HCl (Beckman electrode)	25.0	−0.486	13.708

Apparent Activity Coefficients. These were calculated from the solvent titrations by Levy's (1958) procedure and are listed in Table I. This method serves also to check the acid or alkali error of the glass electrode, *i.e.*, standardizes the electrode system at extreme pH values. The Leeds and Northrup miniature electrode showed an increase in the apparent $-\log \gamma_{H^+}$ values below pH 1.9, with the deviation being smaller at higher ionic strengths and virtually absent in 5 M Gd·HCl.[1] However, the deviation was small, amounting for example to 0.04 at pH 1.4 and 0.05 ionic strength. The Beckman glass electrode gave constant values to 1.4, the lowest pH tested. In the alkaline region, from pH 11.6 to 12.4, the Leeds & Northrup electrode showed again a small decline in the apparent $-\log (K_W/\gamma_{OH})$ value, amounting to 0.01–0.02, whereas the Beckman electrode had no alkaline error in this pH range.

Apparent Hydrogen Ion Activity and the Apparent pK of the Guanidinium Ion in 5 M Gd·HCl at 25°. These were determined as described by Nozaki and Tanford (1967a). There was an appreciable difference between the apparent $-\log \gamma_{H^+}$

[1] Gd·HCl = guanidine hydrochloride.

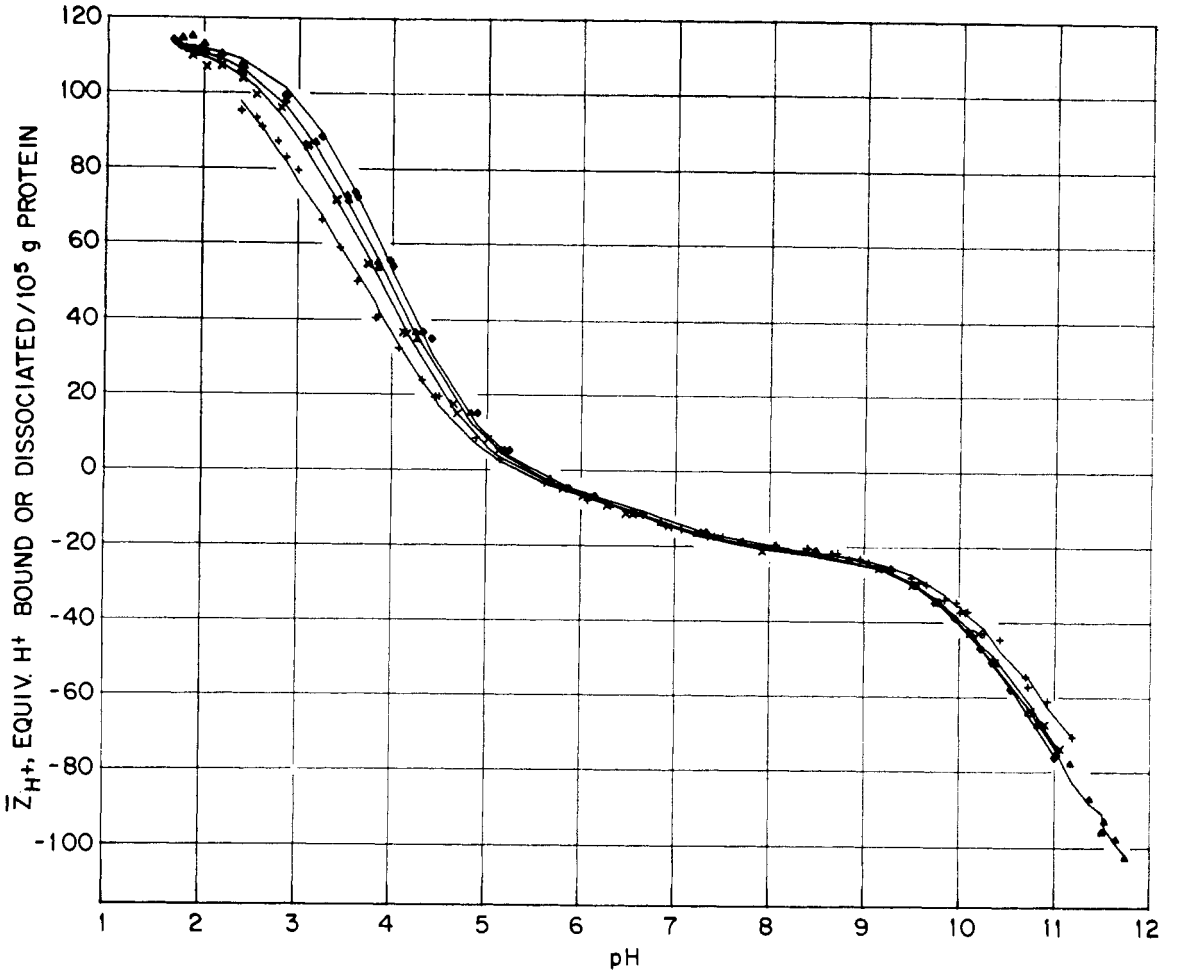

FIGURE 1: Titration curves of bovine fibrinogen at 0.05 (+), 0.15 (×), 0.30 (Δ), and 0.60 (◆) ionic strength. Experimental points and calculated curves plotted by the Calcomp plotter.

values obtained with the two electrode systems and a similar difference was found between the apparent pK's (see Table I). It appears that in this solvent the pH scale of the Leeds & Northrup electrode is situated 0.12 unit more acidic than that of the Beckman electrode. Both $-\log \gamma_{H^+}$ and the apparent pK value determined with the Beckman electrode are very close to the ones reported by Nozaki and Tanford (1967a).

Apparent pK of Formaldehyde. It was estimated by titrating formaldehyde solutions of 8.83, 4.41, 2.20, and 1.04 M concentration, containing 0.3 M KCl, using the Leeds & Northrup miniature electrodes at 25°. The apparent pK varied very slightly with the degree of titration (to about 0.02 unit) and to a larger extent with the initial formaldehyde concentration. The apparent pK extrapolated to 12.86 at zero formaldehyde concentration, a value very close to the one determined by Levy (1934) at 30°. In the calculation of base bound to formaldehyde an appropriate apparent pK was selected for each of the initial concentrations of formaldehyde.

Calculations. The amount of free acid or alkali was calculated at each pH with the apparent H^+ or OH^- activity coefficients of the particular titration conditions. The titration curves were then expressed as equivalents of H^+ bound or dissociated from 10^5 g of fibrinogen. This arbitrary weight was used because the molecular weight of fibrinogen is not definitively established. The equivalents of H^+ bound or dissociated were transformed into average net proton charge, $\bar{Z}_{H^+}$, assuming that the point of zero net proton charge is at the isoelectric point of the protein. The latter is at pH 5.5, according to the electrophoretic experiments of Seegers *et al.* (1945) and Mihalyi (1950). The choice of the above reference point was somewhat arbitrary, but it was considered more realistic for the evaluation of the electrostatic effects.

Results

Isoionic Point. Isoionic fibrinogen was prepared by passing a 2% solution of the protein in 5 M urea through a mixed-bed resin column (Dintzis, 1952) equilibrated with the same solvent. This solvent was used because fibrinogen is insoluble in water in the salt-free state. The protein containing eluate was collected and its pH and conductivity were measured. To avoid CO_2 absorption, the operations were performed, as far as possible, in an argon atmosphere. Five independent experiments gave an average pH of 6.62 ± 0.04. The conductivity of fresh solutions ranged from 2.6×10^{-6} to 3.0×10^{-6} mho, a value only slightly higher than that of a freshly deionized urea solution. The same isoionic point was obtained whether

a neutral solution, fibrinogen at pH 2, or at pH 10.3 was passed through the deionizing column. The isoionic point appears to be more than 1 pH unit higher than the isoelectric point. Urea is known to shift the pH of protein solutions to higher pH values. With 3.3 M urea, added to fibrinogen in 0.3 M KCl, over a wide pH range, this effect was of the order of 0.1 pH unit (Mihalyi, 1954). An experiment was performed to evaluate it also with isoionic fibrinogen: The isoionic protein in 5 M urea solution was diluted with freshly deionized water to 2.5 and 1.25 M concentration. The pH values of the three solutions were 6.69, 6.67, and 6.66, showing that the alkaline shift caused by urea is negligible in this case.

The effects of urea on hydrogen ion activities and dissociation equilibria are complex and ill understood. Large effects may occur; however, the small shifts seen in the above experiments are not unreasonable in view of experiments on model compounds (Donovan *et al.*, 1959; Bull *et al.*, 1964), and the determined isoionic point in urea is probably within 0.2 unit of that in water.

Effect of Ionic Strength on the Titration Curves. The titration curves of fibrinogen at 0.05, 0.15, 0.30, and 0.60 ionic strengths are shown in Figure 1. At each ionic strength at least two independent experiments were performed, with different fibrinogen preparations having slightly different initial net charges. The calculated curves and the experimental points were plotted by the Calcomp plotter (from California Computer Products, Inc., Anaheim, Calif.).

Fibrinogen precipitates in certain pH ranges and the range of precipitation also depends on the ionic strength. A qualitative picture of the solubility relationships is given in Figure 2. Fibrinogen precipitated by acid does not redissolve, or dissolves to a very slight extent, upon neutralization. No attempt was made to demonstrate reversibility of the titration in the range of precipitation, because equilibrium is reached very slowly with the insoluble material. Fibrinogen is unfolded readily at pH values lower than 5 (Mihalyi, 1965), therefore, the titration in this region pertains to the acid-denatured molecule. The titration is reversible in the range of stability of the molecule, from pH 5 up to about pH 10 and above this within the time limits imposed by the rapidly increasing denaturation rates. With the 2-min equilibration time allowed in the latter range, it follows from the rates of denaturation determined as a function of pH that 6% of the protein was denatured at pH 11 and about 25% at pH 11.74 which was the highest pH reached in these studies. Thus the curves from pH 5 to 11 are those of the native material. The few points above pH 11 are mixtures of native and denatured protein in variable proportions, but with preponderance of the native form. The back-titration from about pH 12 shows a pronounced hysteresis. Also, the denatured protein precipitates when approximately pH 10 is reached.

The curves do not have a well-defined acid end point, although they seem to level off at about $\bar{Z}_{H^+} = 114$. The errors involved at low pH are such that extension to the plateau region is not profitable. Assuming an error of ± 0.005 pH unit in the pH measurements, and a similar one in the determination of activity coefficients, the calculated error in $\bar{Z}_{H^+}$ under our conditions of titration, is ± 3.1 at pH 2 and ± 5.3 at pH 1.7, the lower limit of our measurements. The reproducibility of the curves is very good above this pH range, approximately ± 1.0 equiv of bound $H^+/10^5$ g of protein in the steep part of the curve and ± 0.3 in the less

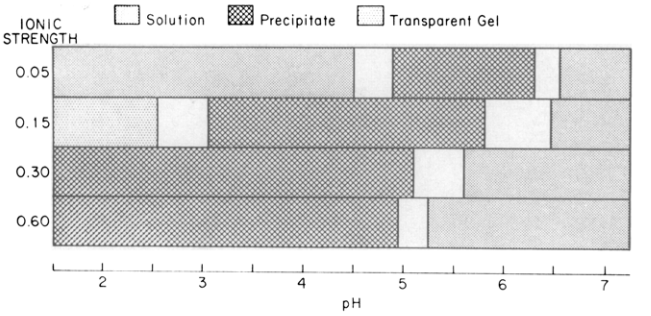

FIGURE 2: Schematic representation of the solubility of fibrinogen as a function of ionic strength and pH.

steep part of the curve between pH 6 and 9. It is noteworthy that the curves above pH 6 and 0.15 ionic strength practically coincide.

Effect of Temperature on the Titration Curves. The titration curves of fibrinogen in 0.3 M KCl at 9.55, 25.0, and 38.0° are shown on Figure 3. The value of $\bar{Z}_{H^+}$ for the same amount of total alkali added differs by no more than 0.08 at the three temperatures in all but the last two samples at the highest pH values. Even here, the largest difference is only 0.6 equiv. Therefore, the apparent heats of ionization were calculated directly from the pH values of the same solution at the different temperatures with the equation given by Wyman (1939)

$$\Delta H = 2.303R \frac{T_1 T_2}{T_2 - T_1}(\mathrm{pH}_2 - \mathrm{pH}_1) \qquad (1)$$

where ΔH is the apparent heat of ionization, R the gas constant, T the absolute temperature, and the subscripts 1 and 2 refer to the two different temperatures. The variation of ΔH with $\bar{Z}_{H^+}$ for the two temperature intervals is shown in Figure 4. The curves show very distinctly the three regions characteristic for the carboxyl, imidazole, and amino group titration. ΔH at the small plateau is 6600 cal and the pH corresponding to this is 6.80–6.85. Both these figures are characteristic for imidazole titration. The fairly wide transition zones, on either side of the plateau, reveal considerable overlapping with the titration of neighboring groups. Since the transitions are nearly linear, their midpoints can be used as approximate border lines between the titration of imidazole and carboxyl groups on the acid side, and imidazole and amino groups on the alkaline side.

Effect of 5 M Gd·HCl on the Titration Curve. The titration curve of fibrinogen in 5 M Gd·HCl is shown in Figure 5. The data obtained with three different fibrinogen preparations and with both electrode systems are included in this figure, with the proper shift of the pH values of the Leeds & Northrup electrode to match the pH scale of the Beckman electrode. At the extreme pH values, the amount of bound acid or alkali was in very good agreement with the two electrodes, provided the apparent H^+ activity coefficient, or pK of guanidine, determined with the corresponding electrode was used in the calculations. This appreciably increased the confidence in the results. The curve showed a very sharp acid end point at $\bar{Z}_{H^+} = 113.5$, showing that the conditions of titration at high acidity are more favorable in this solvent

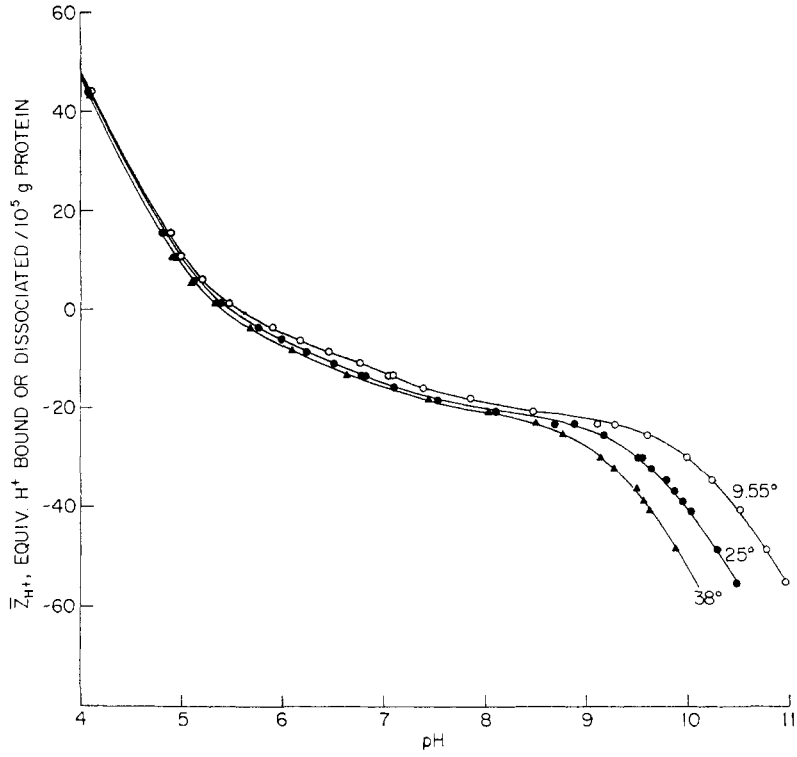

FIGURE 3: Titration curves of fibrinogen at 0.30 ionic strength, at three different temperatures.

than in KCl solutions. There is an indication of another stoichiometric point at the alkaline end of the curve, at $\bar{Z}_{H^+} = -110$ to -120.

The titration was completely reversible from the acid end to approximately pH 10, at which point the back-titration was stopped. There was no such reversibility from the highest pH of these experiments of approximately 11.5. Back-titration showed the presence of about three new groups with a pK in the neighborhood of 8. It is highly unlikely that this was caused by CO_2, which was taken up from the atmosphere in spite of all the precautions that were taken. Similar phenomena were observed by Nozaki and Tanford (1967b) with the titration of ribonuclease in 6 M Gd·HCl and were attributed to the β-elimination reaction of the disulfide bonds described by Bohak (1964). Fibrinogen has about 10 disulfide bonds/10^5 g, and apparently 30% of these suffered the β-elimination reaction. The titration in the forward direction was performed as fast as possible, taking about 30 min from pH 10 to 11.5. Perhaps, during this short time interval the reaction could not progress to an appreciable extent. The stoichiometry at least did not require any additional groups; if anything

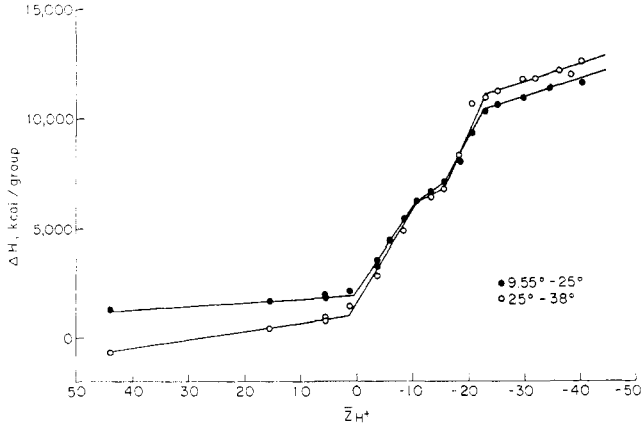

FIGURE 4: The apparent heats of ionization plotted as a function of the net proton charge of fibrinogen.

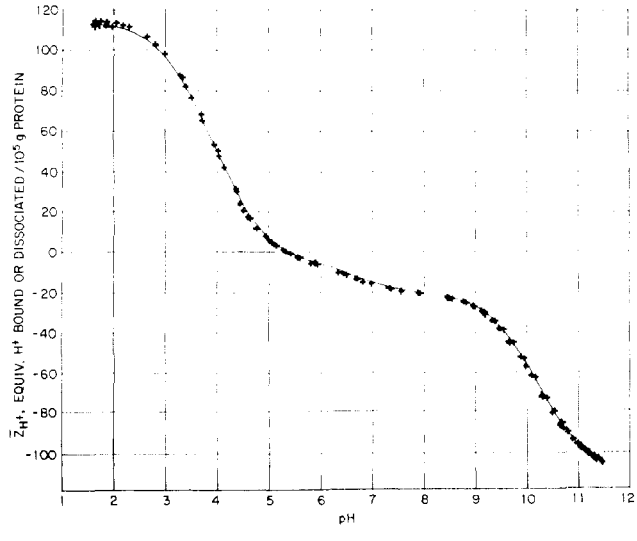

FIGURE 5: Titration curve of fibrinogen in 5 M Gd·HCl. Experimental points and calculated curve were plotted by the Calcomp plotter.

TABLE II: Stoichiometric Points of the Titration Curves.

Nature of Titration	Position of the Stoichiometric Point	Groups Titrated from the Acid End Point to the Stoichiometric Point: Nature of Groups	Number of Groups
Titration in 5 M Gd·HCl	113.5	Acid end point	0
Thermal method (ΔH *vs.* $\bar{Z}_{H^+}$ curve)			
Midpoint of the first transition	−5	COOH	118.5
Midpoint of the second transition	−20	COOH + imidazole	133.5
Isoionic point[a]	−11.5	Imidazole + NH_2 + guanidino	125.0
Titration in 8.08 M formaldehyde	−85	COOH + imidazole + NH_2	198.5
Titration in 5 M Gd·HCl	−115	COOH + imidazole + NH_2 + phenolic OH	228.5

[a] The total number of cationic groups is equal to the number of groups titrated from the isoionic point to the acid end point (Cannan *et al.*, 1941). Additional data: number of ϵ-NH_2 groups from formol titration difference curve, 63.0; number of phenolic OH groups from spectrophotometric titrations (Mihalyi, 1968), 29.1; and number of α-NH_2 groups from N-terminal analysis (Blombäck and Yamashina, 1958), 1.2.

it was short by one to two groups. The mixture was allowed to stand 20 min at the high pH before the back-titration was started and probably the reaction occurred mostly during this period.

Effect of Formaldehyde on the Titration Curves of Fibrinogen. Figure 6 shows these curves in 0.3 M KCl in the presence of 2.02, 4.04, and 8.08 M of formaldehyde. A pronounced inflection point is apparent at $\bar{Z}_{H^+} = -85$. For reference the curve obtained in 0.3 M KCl in the absence of formaldehyde is also included. The difference curves between the titrations with and without formaldehyde are shown in Figure 7. A well-defined plateau whose height increases slightly with the formaldehyde concentration and reaches a value of 63.0 at 8.08 M concentration of the latter, is observed with these. This number must be very close to the actual number of ϵ-amino residues (Kekwick and Cannan, 1936). The α-amino groups are titrated at a lower pH range, therefore, they are not included in this number.

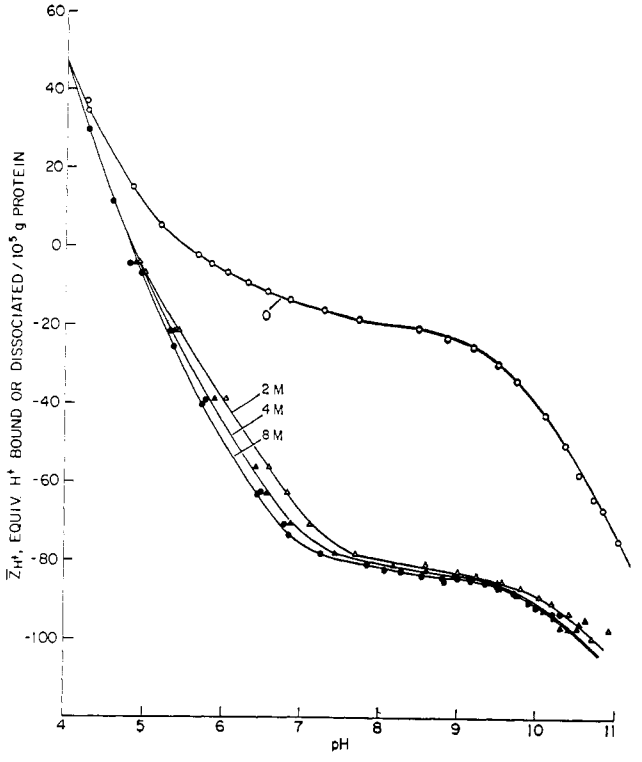

FIGURE 6: Titration curves of fibrinogen in the absence and in the presence of 2, 4, and 8 M formaldehyde.

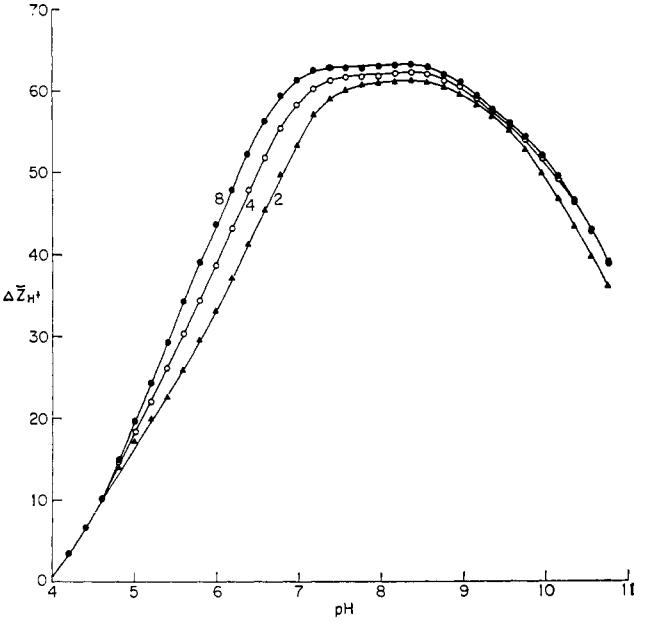

FIGURE 7: Difference curve between the titration in the absence and in the presence of 2, 4, and 8 M formaldehyde.

TABLE III: Parameters, Fixed or Obtained from Curve Fitting by Computer, of the Alkaline Section of the Titration Curve at 0.30 Ionic Strength.

Mode of Computation	Parameters				
	N_3	pK_3	N_4	pK_4	w_2
Individual	106.8	10.28	*45.0*[a]	*13.5*	0.0113
	104.4	10.27	*45.0*	*12.0*	0.0114
	93.4	10.20	*45.0*	11.07	0.0124
N_3 subdivided	*93.4* = *64.3*	10.23			
	93.4 = *29.1*	*10.43*	*45.0*	10.49	0.0134
All in one group	101.9	10.28	*45.0*	*13.5*	0.0106

[a] Quantities set in italic type were used as fixed parameters in the computations, the others were determined by the computer.

Fitting of the Titration Curves. The assignment of the various segments of the titration curve to the various ionogenic groups of the protein is a well-established procedure (see, for example, Tanford, 1962). A preliminary counting of the groups was made on the basis of the stoichiometric points obtained in the various titrations described in the preceding sections. The stoichiometric points are listed in Table II. With simple arithmetic the number of groups in each class can be determined. The α-COOH and α-NH_2 are too small a number to be detected as individual classes. The former are included with the total COOH and the latter in most part with the ϵ-amino and tyrosyl residues. The following number of groups were obtained: carboxyl, 119; imidazole, 15; ϵ-amino, 63; and guanidyl, 45. To these, the 29 tyrosine residues found by spectrophotometric titration should be added. The molecule does not contain SH groups (Bagdy *et al.*, 1948).

The detailed fitting of the titration curves at various ionic strengths was performed by the IBM 360 computer. The general equation of the titration curves was assumed to have the form

$$\bar{Z}_{H^+} = (\bar{Z}_{H^+})_{max} - \Sigma^i N_i \frac{K_i'}{[H^+] + K_i'} \qquad (2)$$

and

$$K_i' = K_i^0 e^{2w\bar{Z}_{H^+}} \qquad (3)$$

$(\bar{Z}_{H^+})_{max}$ in these equations is the maximum positive charge of the molecule, *i.e.*, the number of hydrogen ions bound from pH 5.50 to the acid end point; N_i is the number of groups titrated in the ith class of titratable groups and K_i' is their apparent dissociation constant; $[H^+]$ is the hydrogen ion concentration; K_i^0 is the intrinsic dissociation constant of the ith class; and w is the electrostatic interaction factor. $(\bar{Z}_{H^+})_{max}$ was treated as a constant, with its value fixed to +113.5 by the end point of the titrations in 5 M Gd·HCl. From the five classes of groups mentioned in the preceding paragraph, the ϵ-NH_2 and tyrosyl residues overlap to such an extent that their resolution was not possible. These were, therefore, combined in a common class, reducing thereby the number of classes to four. The number of arginine residues cannot be estimated directly from the titration curves. The indirect estimate, from the sum of cationic groups, gave 45.0 as their number, and this was used as a fixed quantity for N_4 in all the computations. The computer was programmed to find the number of groups and the intrinsic pK in each of the classes, together with the electrostatic interaction term w, on the basis of eq 2 and 3. The N_i and pK_i values were constrained to very generously taken reasonable ranges derived from the stoichiometric points given in Table II. From the pH-stability relationship of the molecule (Mihalyi, 1965) it appears that fibrinogen is expanded on the acid side of the isoelectric point and shows only minor signs of conformation change above this point, if alkaline denaturation is avoided. Therefore, in the computation one w value was allowed for the carboxyl region (w_1) and another (w_2) for the rest of the titration curve.

The computations yielded unequivocal solutions for the carboxyl and imidazole regions, but the third segment, comprising the ϵ-amino and phenolic groups, was to a large extent undefined. This was undoubtedly because of the incomplete titration of these groups. The titration curves do not show any indication of a stoichiometric point in this range, and indeed, from the data obtained, it appears that only about two-thirds of the residues of the third class are titrated up to the points of the highest alkalinity used.

A series of computations clearly demonstrated the latitude of the parameters describing the third and fourth class of groups. Table III presents the combination of parameters, some of them fixed and others computed, which all gave practically indistinguishable calculated curves, fitting the experimental data to approximately ±1.0 equiv. For the sake of brevity, only the data obtained at 0.3 ionic strength are listed. The data show that decreasing pK_4 lowers N_3, *i.e.*, the titration of the arginine residues contributes more and more to the groups titrated in the third class. Thus, fixing pK_4 to a reasonable value defines N_3; alternatively, a given N_3 value sets the value of pK_4. Since the customary assumption of pK_4 > 12 gave unreasonably large values of N_3, the second alternative was considered more applicable to this problem. The sum of the lysine and tyrosine residues, from the formol

TABLE IV: Parameters of the Titration Curves Determined by the Computer.

Parameters	Ionic strength					Mean Values
	0.05	0.15	0.30	0.60	5.00[a]	
N_1	118.92	119.35	118.79	119.22	119.02	119.06
pK_1	4.037	4.117	4.179	4.210	4.114	
N_2	14.51	15.70	15.20	15.44	15.44	15.46
pK_2	6.649	6.656	6.604	6.799	6.690	
N_3	93.40[c]	93.40[c]	93.40[c]	93.40[c]	92.63[e]	93.40[c]
pK_3	10.290	10.244	10.200	10.277	10.086	
N_4	45.00[c]	45.00[c]	45.00[c]	45.00[c]	45.00[c]	45.00[c]
pK_4	>13[d]	10.921	11.071	10.608	>13	
w_1	0.0101	0.0071	0.0063	0.0048	0.0028	
w_2	0.0140	0.0133	0.0124	0.0124	0.0022	
Root-mean-square deviation[b]	1.2	1.2	1.4	1.2	0.7	
Maximum deviation[b]	4.1	2.7	5.0	4.0	2.3	

[a] In 5 M Gd·HCl. [b] Deviation of the experimental $\bar{Z}_{H^+}$ values from the calculated ones. [c] Values used as fixed parameters. [d] Since class three was titrated to about 50% in this experiment, the normalization of the arginine residues may be only apparent, the result of the incompleteness of the titration. [e] This figure includes 1.2 groups with a pK of about 7.5.

and spectrophotometric titrations, was 92.2, to which 1.2 was added for the α-NH_2 groups (the latter calculated on the basis of Blombäck and Yamashina's (1958) model). Thus, with N_3 fixed to 93.4 the curves were recalculated. An excellent fit to the experimental points was obtained, as shown in Figure 1. In view of the aforementioned facts this fit is by no means a proof of the correctness of the parameters of class three and four. However, the fact that they are in harmony with all the data collected, including the analytical ones, makes them probably the best choice. Therefore, these data are used in Table IV, where all parameters derived for the entire titration curve, at each ionic strength, are assembled. The value of pK_4 came to approximately 11 with this procedure, which is much lower than expected. An attempt was made to improve the situation by subdividing class three into its two components. Namely, the spectrophotometric titrations gave 10.43 as pK_0 of the tyrosyl residues and this is about 0.2 unit higher than the average pK_3 computed here. The results, however, showed no appreciable change in pK_4.

In the previous computations the curve at each ionic strength was analyzed individually. When pK_4 was fixed to 13.5 or 12.0 the N_3 values increased from 86 to 107 with the successive curves at increasing ionic strength. In the same time, pK_3 was nearly constant at 10.25 ± 0.02. Since N_3 should be the same in all the runs and pK_3 appeared to be nearly invariable, computations were performed with the experimental points, at all four ionic strengths, taken together and stipulating equal pK_3, pK_4, N_3, and N_4 values with all of them. The only variable to distinguish the curves at various ionic strengths was w_2 which was allowed to assume an individual value at each ionic strength. These computed curves were indistinguishable from those computed individually. It was hoped that by increasing the number of experimental points in this region, the computations will give a more reasonable set of parameters. However, as indicated in the table, with pK_4 fixed to 13.5, the value of N_3 was still too high. Therefore, this method is not more advantageous than that of the individual computations and does not circumvent the use of a fixed quantity for N_3.

The computation of the titration curve in 5 M Gd·HCl was performed in a similar fashion. A better fit of the acidic segment was obtained by increasing $(\bar{Z}_{H^+})_{max}$ to 115.0. The alkaline segment was better defined than with the curves obtained at various ionic strengths. A unique set of parameters was found to describe it accurately with pK_4 so high that the fourth class did not contribute practically at all, even at the highest point of alkalinity reached. Moreover, N_3 was very close to the value accepted as the most reasonable one for the curves in KCl solutions. Comparison of the calculated and experimental curves definitely showed the presence of 1–1.5 groups titrating with a pK of approximately 7.5, and indeed, a much better fit was obtained when 1.2 equiv were taken out of the N_3 class and the above pK was assigned to them. The w values were very small and not too different in the acidic and alkaline section of the curves, so that equally good fit was obtained by using a single w for the entire curve.

Discussion

Stoichiometry. The stoichiometric figures derived from the titrations of the native fibrinogen in salt solutions and of the denatured protein in 5 M Gd·HCl were nearly identical. Therefore, their averages were calculated. These are compared with the analytical values of Henschen and Blombäck (1964) and of Mihalyi *et al.* (1964) in Table V. The agreement with the basic groups is very good, except with the number of ϵ-NH_2 groups given by Henschen and Blombäck, which is too low by approximately five residues. The situation with the free carboxyl groups is less satisfactory and the cause of this is undoubtedly the difficulty with the estimation of the amide groups. Fibrinogen contains 8.2 moles of sugar-

TABLE V: Comparison of the Number of Groups Found by Analysis with the Number Found by Titration.

Nature of Groups	Residues/10^5 g of Fibrinogen: Analytical Data: Henschen and Blombäck (1964)	Analytical Data: Mihalyi *et al.* (1964)	Titration
α-COOH[a]	1.8	1.8	
γ,δ-COOH	114.7	97.0	
Sialic acid[b]	2.9	2.9	
Total COOH	119.4	101.7	119.0
Imidazole	15.3	16.6	15.5
α-NH_2[a]	1.2	1.2	1.2
ϵ-NH_2	58.4	63.5	63.0
Guanidyl	44.5	44.9[c]	45.0
Total cationic	119.4	126.2	124.7
Phenolic	30.9	29.2	29.2

[a] Values calculated on the basis of the six-chain model of fibrinogen (Blombäck and Yamashina (1958). [b] From Mester (1968). [c] In the original communication a larger value was given, obtained by extrapolation to zero hydrolysis time. Because arginine is not destroyed during hydrolysis in most proteins, this was recalculated as the average of values obtained with increasing hydrolysis time.

bound nitrogen/10^5 g of protein (Mester, 1968) and this yields a variable amount of ammonia during the amide nitrogen determinations. Thus, the analytical amide figures tend to be too high, causing the calculated free carboxyl figures to be too low. At the moment, the titration data appear to be much more trustworthy and the chemical estimate of amide groups clearly needs reinvestigation. Fibrinogen also contains 2.9 moles of sialic acid/10^5 g of protein (Mester, 1968), which with a pK of 2.6 should be almost completely titrated at the low pH end of the titration curves. These are, therefore, included in the total number of carboxyl groups determined by the titrations.

The excellent agreement of the number of arginine residues obtained by analysis and from the titration curves is an additional proof of the correctness of the isoionic point determined in this work. Were the isoionic and isoelectric points identical, the calculation from the titration curves would have yielded 34 arginine residues, a value much too low, even with allowance for the errors involved in these computations.

In conclusion, it should be pointed out that the complete stoichiometric analysis of the titration curves required a combination of nearly all the available data. Especially, the curves at various ionic strengths would have been insufficient in themselves to resolve the alkaline section of the curves. For the latter, the titrations in formaldehyde and 5 M Gd·HCl proved especially valuable.

Electrostatic Effects. It appears from Table VI that the

TABLE VI: Experimental and Calculated Electrostatic Interaction Factors.

Ionic Strength	Experimental w: Acid Range	Experimental w: Neutral Range	Calculated w: Spherical Model (eq 4)	Calculated w: Cylindrical Model (eq 5)
0.05	0.0101	0.0140	0.0368	0.0192
0.15	0.0071	0.0133	0.0269	0.0126
0.25[a]		0.0133	0.0235	0.0105
0.30	0.0063	0.0124	0.0223	0.0099
0.60	0.0048	0.0124	0.0187	0.0080
5.00[b]	0.0028	0.0022	0	0

[a] From spectrophotometric titrations (Mihalyi, 1968). [b] In 5 M Gd·HCl.

experimental w values are much smaller in the carboxyl region than in the neutral and alkaline regions of the titration curves. Regardless of the model assumed (*i.e.*, treating w as an empirical parameter), this can be interpreted as an indication of the expansion of the molecule (Tanford, 1955) in the acid range. Optical rotation data (Mihalyi, 1965) are consistent with such an interpretation. In the acidic region, the experimental w shows the expected variation with the ionic strength. Above the isoelectric point, however, w is nearly insensitive to the latter.

According to Hall and Slayter (1959) the fibrinogen molecule consists of three spherical formations disposed in a linear array. The two nodules at the extremities are 65 Å, the middle one 50 Å in diameter and the center of two adjacent nodules is separated by a distance of about 200 Å on the electromicrographs, possible more in solution. The electrostatic interaction between any two of the connected spheres is small because of the fairly large distances involved. The relative proportion of the groups titrated in the intermediate sections is equally small, so their contribution may be neglected. Therefore, from the electrostatic point of view, fibrinogen can be approximated by isolated spheres with the diameter of the nodules. Table VI contains the w values calculated for a sphere of 61-Å diameter, at the ionic strengths of the titrations with

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \qquad (4)$$

Where e is the electron charge, D the dielectric constant of the solvent, k the Boltzmann constant, T the absolute temperature, b the radius of the protein, a the radius of closest approach of counter ions, and κ is the reciprocal thickness of the ion atmosphere surrounding the protein molecule. The latter, in water, at 25°, is given by the relationship $\kappa = 3.87 \times 10^{-7} I^{1/2}$, where I is the ionic strength. The diameter used corresponds to an unhydrated sphere of 100,000 molecular weight, with a partial specific volume of 0.72. This value is very close to the average diameter of the spherical nodules in Hall's model.

Alternatively, a cylindrical model may be used and w calculated with Hill's (1955) equation

$$w = \frac{e^2}{DkTL}\left[\frac{K_0(\kappa a)}{(\kappa a)K_1(\kappa a)} + \ln\frac{a}{b}\right] \quad (5)$$

In this equation, K_0 and K_1 are modified Bessel functions of the second kind, L is the length and b the radius of the cylinder, a is the radius of closest approach, and the other symbols have the same meaning as in the previous equation. The dimensions of Hall's model were used: 475 × 65 Å; and since these refer to the anhydrous molecular weight of 340,000, the calculated values were multiplied by 3.4 to make them comparable with the spherical model and the experimental data.

Both calculations are based on the smeared charge model of the protein; which, in some instances at least, may require serious refinements. However, it proved to be applicable in a number of cases (see the discussion of Tanford, 1962); moreover, it is probably closer to reality with larger proteins, with many charged groups, than with smaller ones. As expected, the cylindrical model gives only about half the electrostatic effect of the spherical one, but the relative effect of the ionic strength is approximately the same in both cases. Comparison of the experimental values for the neutral range with the calculated ones shows that the cylindrical model might be more appropriate. However, the absence of ionic strength effect cannot be explained with either model and it is doubtful that a discrete charge model could be of more help in this respect. Explanations, based, for example, on an ionic strength dependent conformational change, would be purely speculative in the absence of additional physicochemical data.

The electrostatic interaction term, contrary to expectations, does not vanish in 5 M Gd·HCl. Similar findings were reported with lysozyme (Donovan *et al.*, 1960), ribonuclease (Cha and Scheraga, 1960; Nozaki and Tanford, 1967b), paramyosin (Riddiford and Scheraga, 1962), and α-lactalbumin (Robbins *et al.*, 1967). According to Nozaki and Tanford the larger than expected w of ribonuclease in guanidine has its explanation in the heterogeneity of the pK's of the carboxyl and tyrosyl groups. When this factor was introduced into their calculations the whole curve could be fitted with an electrostatic factor equal to zero. It is significant that this anomaly with other proteins was limited to, or more pronounced in the titration region of the carboxyl groups. For example, w was 0 with the spectrophotometric titration of the tyrosyl residues of fibrinogen in 5 M Gd·HCl (Mihalyi, 1968), whereas with the whole titration curve, as reported above, an apparent electrostatic effect was definitely present. Thus, the tyrosine residues in themselves are homogeneous, but the third class of groups, which comprises both tyrosyl and lysine residues, is obviously heterogeneous. These phenomena will be discussed further in connection with the theoretical titration curves generated by the computer.

Intrinsic Dissociation Constants. The ionization constants evaluated by the computer are given in Table IV. For these to be true thermodynamic constants the reaction should be reversible, and to be intrinsic, beside correcting for electrostatic effects, their titration should not be coupled to conformation changes of the protein. These criteria are certainly not met by the carboxyl titration, which occurs on unfolded protein, moreover, the degree of unfolding is a function of the net charge of the molecule (Mihalyi, 1965). Nevertheless, the pK's obtained are well within reasonable range, showing that the conformational change, which accompanies the increase of the net charge of the once unfolded protein, has a small change in free energy.

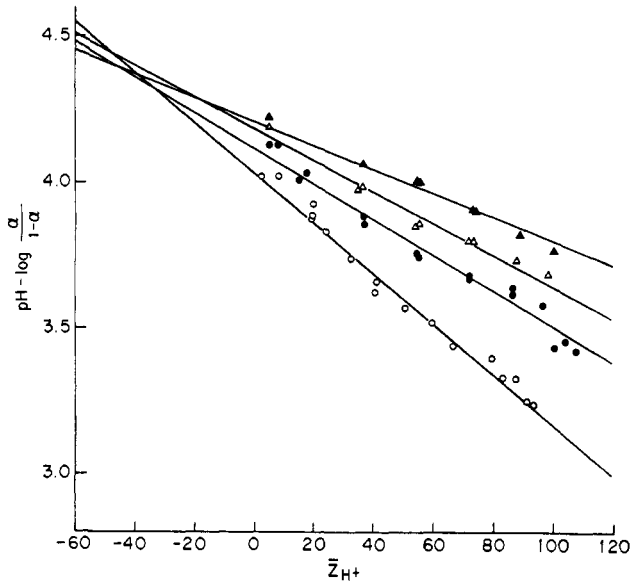

FIGURE 8: Logarithmic plot of the carboxyl region of the titration curves of fibrinogen at 0.05 (○), 0.15 (●), 0.30 (△), and 0.60 (▲) ionic strength.

The apparent pK_0 values of the carboxyl groups show a small but systematic increase with the ionic strength. The discussion of this effect is facilitated by a plot of the data on the basis of the rearranged titration equation

$$\mathrm{pH} - \log\frac{\alpha}{1-\alpha} = \mathrm{p}K_0 - 0.868w\bar{Z} \quad (6)$$

where α is the fraction titrated. A plot of [pH − log (α/(1 − α))] *vs.* $\bar{Z}$ should give a straight line with the slope equal to −0.868 w and pH − log (α/(1 − α)), at $\bar{Z}$ = 0, equal to pK_0. This is shown on Figure 8, where the lines were drawn with the parameters supplied by the computer. These do not intersect at $\bar{Z}_{H^+}$ = 0, as they should, if the apparent pK's were influenced only by the general net charge effect. Among others, the following factors may be responsible for this: the effect of ionic strength on the intrinsic pK's (Tanford, 1962), inadequacy of the smeared charge model (Tanford, 1957; Nagasawa and Noda, 1968), and binding of other ions than hydrogen. The first two factors are certainly involved and could be sufficient to explain the effect. However, binding of anions should be considered also. The net charge of the protein was calculated assuming no other ions bound except hydrogen and assuming a zero net charge at the isoelectric point. Since the isoionic point was found more alkaline than the isoelectric point, anions are definitely bound. Their number must increase with an increase of the anion concentration and also with that of the positive net

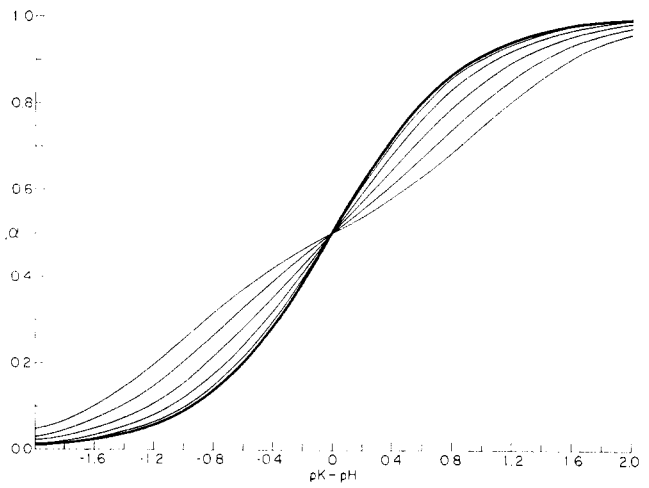

FIGURE 9: Calculated titration curves, with two subclasses of equal weight and with intrinsic $\omega = 0$. The difference of pK's of the subclasses was increased in increments of 0.4, from 0 to 2.0, with the successive curves.

charge on the protein. In consequence, not only the zero point of the abscissa will be shifted to a different value for each of the lines, but also the actual net charge increments will be diminishing with increasing $\bar{Z}_{H^+}$. This should result in steeper slopes, *i.e.*, higher values of the true w. The low apparent w values discussed in the preceding section may be caused partly by this effect. The resolution of all these problems is not possible without accurate data on the binding of chloride ions, which at the present are nonexistent.

If the isoionic point proves to be correct, the zero point of the $\bar{Z}_{H^+}$ scale should be shifted by 11.5 in the negative direction on all the figures which have $\bar{Z}_{H^+}$ as their ordinate. The pK_0 values on the alkaline side will be shifted also by $0.868\ \Delta\bar{Z}$, where $\Delta\bar{Z}$ is the above net charge difference between the assumed isoelectric and the isoionic point. This, with the present w values, amounts of 0.12–0.14 unit, not much larger than the expected experimental error. The shifts of the carboxyl pK's will be larger, but in the absence of excessive chloride binding, the pK's will still remain in their reasonable range.

The pK_0 values found for both the imidazole and the ϵ-amino groups appear to be within their normal range, moreover, they show the same peculiar variation with the ionic strength as found by Neuberger (1937) with the dissociation of a cationic acidic group of a dipolar ion. The variation, however, is small and it may not even be significant with the precision of the calculations. It should be borne in mind that pK_0 of the third class is some combination of the pK's of both the ϵ-amino and phenolic groups, with the former predominating in virtue of their larger number. Since the spectrophotometric titrations showed a higher pK for the tyrosyl residues than the figure comprising both kind of groups, the actual value for the ϵ-amino residues should be lower than the combined value.

The pK obtained for the arginine residues is low but not unreasonable. In arginine peptides (Edsall, 1943) the pK of the guanidino group is approximately 12.4. In most proteins it appears to be even higher; however, in a few cases reliable estimates showed much lower values, ranging from 11.5 to 11.9 (Tanford and Epstein, 1954; Tanford and Wagner, 1954; Léonis and Li, 1959; Riddiford and Scheraga, 1962). Clustering of positive charges may account for the lowering of the average pK of these groups. This is clearly seen in α-corticotropin (Léonis and Li, 1959), where most of the lysine and arginine residues are contained in a short sequence.

The intrinsic dissociation constants in 5 M Gd·HCl are also given in Table IV. They are very nearly the same as in KCl solution, as they should be. A point worth mentioning is the normalization of the pK of the arginine residues. With the reservations in order for the data from this technically difficult section of the titration curves, this shows that the low value in the native state is connected with the secondary–tertiary structure and not a particular sequence of the amino acids.

One conclusion which emerges from the discussions of the preceding paragraphs is that all titratable groups of fibrinogen appear to titrate in a normal fashion, or very close to this. The number of groups titrated is the same as the one derived from the amino acid composition of the protein and there are no major anomalies in the pK's of these groups. However, it should be pointed out that the carboxyl groups were titrated in the denatured molecule and there is no assurance that they will titrate in the same way in the native molecule. Experimentally it is very difficult to solve this problem because of the extremely high rate of the acid denaturation and because of the precipitation of fibrinogen under these conditions. Barring any change in the titratability of the carboxyl groups on denaturation, one can state that all the groups titrated are on the surface of the molecule, freely accessible to hydrogen ions.

Binding of Chloride Ions. The difference between the isoelectric and isoionic points clearly indicates binding of anions. This difference, according to the titration curves, corresponds to 11.5 Cl^- bound. Assuming that this occurs at 0.05 M concentration of Cl^-, and that all the cationic groups are binding sites of equal strength, a binding constant of 2 can be calculated. This is within the range of the intrinsic chloride binding constants of free amines, determined by Saroff and Healy (1959). Another indication of chloride binding is the anomalous behavior of the pK of the carboxyl groups and the lower than expected w connected with their titration. The insolubility of the protein in this region presents formidable experimental difficulties for chloride binding studies. Without these, however, a complete interpretation of the titration data is not possible.

Computer-Simulated Titration Curves. The effect of heterogeneity of pK's within one group, on the apparent w values, was investigated theoretically. The simplest case is that of two subclasses of equal weight, with intrinsic $w = 0$. A series of titration curves was generated in the computer, allowing the difference between the pK's to increase from 0 to 2.0, in steps of 0.4. From the curves shown in Figure 9 it is evident that the pK difference should be larger than 0.8 before an indication of two separate classes appears. As expected, the apparent pK of these curves, that is the pH at half-titration of the whole class, was situated at the arithmetic mean of the two individual pK's. The curves were made to coincide at their apparent pK's. When the points were plotted according to eq 6, taking Z equal to α, the curves shown in Figure 10 were obtained. This plot is more sensitive to heterogeneity

TABLE VII: Apparent w Values Calculated with Two Subclasses of Equal Weight and Increasing Separation of Their pK's.

ΔpK	w_{app}
0	0
0.2	0.026
0.4	0.100
0.6	0.219
0.8	0.364

than the usual titration curve, nevertheless, even at ΔpK equal to 0.8 the straight-line relationship is obeyed to within 0.01 pH unit over more than 80% of the titration. Thus, with either plot, the difference between pK's should be over one unit in order to detect heterogeneity. The w value corresponds to the titration of a single group, because α varies from 0 to 1.0. The shape of the curves will be the same with any number of groups titrated, but now the abscissa will span the interval from 0 to N and the calculated w will be equal to the value for one group divided by N. Table VII contains the calculated apparent w values. A plot of these against ΔpK can be used to determine the latter from the former. For example, Nozaki and Tanford found for ribonuclease $w_{app} = 0.020$. Since there are 11 carboxyl groups titrated, this corresponds to $w = 0.22$ with the units used in the table and $\Delta pK = 0.6$, close to the value found by the above authors. For fibrinogen, w_{app} was 0.0028, with 119 groups titrated. If converted into the proper units, this corresponds to $\Delta pK = 0.75$. The latter value is larger than expected, (see Nozaki and Tanford, 1967c), but in view of the limited precision of the experimental w values, not too much significance should be attached to this.

When the distribution of the two subclasses was varied from 1:4 to 4:1, while ΔpK was held at 0.8, smooth titration curves were obtained. The logarithmic plots, however, were deviating more and more from linearity as the ratio of the two subclasses became more unbalanced. The apparent pK's were very close to the weighted mean of the two original pK's.

Curves with five subclasses of equal weight, and with equally distanced pK's, gave linear logarithmic plots over 80 and 70% of the titration span when ΔpK was 0.4 and 0.8, respectively. The apparent w values became very large: 0.68 and 2.21.

With all the previous curves the intrinsic w was assumed to be 0. In the next step, a curve was generated simulating the titration of the carboxyl residues of fibrinogen, but assuming $(\bar{Z}_{H^+})_{max}$ equal to the number of carboxyl groups, which is not too far from reality. It was assumed further that the intrinsic w is 0.013, much larger than the actual values, and the COOH groups are equally divided into two subclasses with ΔpK equal to 0.5. The calculated apparent w was 0.0121, smaller than the intrinsic w. In agreement with this, when the carboxyl region of the actual titration curves was subdivided into two subclasses of equal weight, the fit was as good as with the undivided class and the w values were nearly the same.

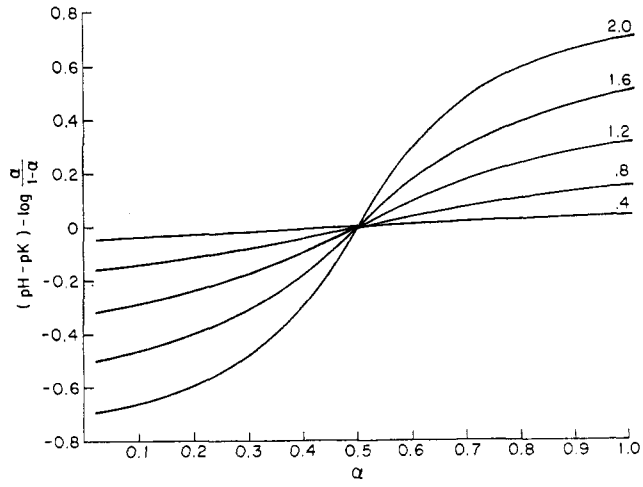

FIGURE 10: Logarithmic plots of the titration curves shown on Figure 9.

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Kinetics of Dinitrophenylation of Amino Acids*

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ABSTRACT: The kinetics of reactions of 1-fluoro-2,4-dinitrobenzene (FDNB) with glycine, proline, and *N*-phenylglycine in water, of FDNB with glycine in acetonitrile–water and dimethyl sulfoxide–water mixtures, and of 2,4-dinitrophenyl phenyl ether (DNPE) with proline and pyrrolidine in 10% dioxane–90% water have been measured as a function of base concentration or solvent composition. Dinitrophenylation of glycine with FDNB is very much faster in dimethyl sulfoxide–water than in ethanol–water or acetonitrile–water mixtures; this may be of practical value in application of dinitrophenylation for analytical or peptide modification purposes. The pH dependence of rates of reactions of FDNB with amino acids is entirely accounted for by the effect of pH on the state of ionization of the amino acids; there is no evidence for base catalysis of the substitutions proper. The reaction of DNPE with proline anion is catalyzed by NaOH, but surprisingly the reaction of DNPE with pyrrolidine is insensitive to base catalysis.

Although the Sanger method of peptide end-group analysis and sequence determination through dinitrophenylation of amino groups with 1-fluoro-2,4-dinitrobenzene[1] (Sanger, 1945; Fraenkel-Conrat *et al.*, 1955) has to a large extent been supplanted by the Edman phenyl isothiocyanate degradation procedure (Schroeder, 1967; Konigsberg, 1967; Edman and Begg, 1967) and by dansylation with 1-dimethylaminonaphthalene-5-sulfonyl chloride (Gray, 1967a,b), dinitrophenylation with FDNB continues to be widely used for purposes of end-group analysis, modification of proteins, and identification of peptide fragments.

Despite the extensive use of this method in biochemical research, the kinetics of reactions of amino acids and peptides with FDNB have not received much attention. The rates of reaction of a few amino acids and peptides with FDNB in water solution were determined by Burchfield and Storrs (1957) and in water under heterogeneous conditions (FDNB was present as a separate liquid phase) by Brouwer *et al.* (1958). In both studies, some attention was given to variation of pH (the pH was varied by only one unit) but the influence of pH has otherwise been little investigated.

In view of the fact that reactions of FDNB with some amines in protic media are catalyzed by base (Bunnett and Randall, 1958; Beale, 1966) while reactions with other amines are not base catalyzed (Bunnett and Garst, 1965a), it was of interest to see if reactions of amino acids with FDNB might respond to catalysis by bases. Particularly if some reactions were base catalyzed and others not, variation in pH might enable selective dinitrophenylation of certain aminoacyl moieties. For this reason, the present investigation of the pH dependence of reaction rates in water solution was undertaken.

Inasmuch as the reactions of amines with nitro-activated aryl halides are much faster in Me_2SO and other dipolar aprotic solvents than in aqueous or alcoholic media (Suhr, 1963, 1964; Kingsbury, 1964), the influence of this factor

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[1] Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNPE, 2,4-dinitrophenyl phenyl ether.