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A THERMOCHEMICAL INVESTIGATION OF THE BINDING OF 12-PHOSPHOTUNGSTIC ACID AND CHLORIDE ION TO BOVINE SERUM ALBUMIN

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ABSTRACT

The reaction of bovine serum albumin (BSA) and several other proteins with 12-phosphotungstic acid (12-PTA) has been investigated by the method of thermometric enthalpy titration. For all proteins tested the enthalpograms indicate that the reaction proceeds in two stages. The reaction of BSA with 12-PTA has been studied as a function of pH from 1 to 5, of ionic strength from 0.10 to 1.0 M in sodium chloride–hydrochloric acid mixtures and as a function of BSA concentration from 0.50 to 20 g l^{-1}. The studies indicate that some chloride (or other anion) binds to or condenses on the surface of the positively charged protein. Other chloride ions are present near the protein only as counter ions in order to satisfy electroneutrality considerations. When 12-PTA is added slowly and continuously to a protein it *first* reacts with the available positive sites to which no anion is *strongly* bound thus:

$$H_nP(Cl)_v^{(n-v)+} + \frac{n-v}{3}PTA^{3-} \rightleftarrows H_nP(Cl)_v(PTA)_{\frac{n-v}{3}} \quad (\downarrow)$$

In the second reaction stage the chloride ion is displaced from the protein precipitate by additional 12-PTA.

$$H_nP(Cl)_v(PTA)_{\frac{n-v}{3}} + \frac{v}{3}PTA^{3-} \rightleftarrows H_nP(PTA)_{\frac{n}{3}}(\downarrow) + vCl^-$$

INTRODUCTION

The interaction of small inorganic ions and larger organic ions with proteins has been a subject of considerable interest[1–4]. The principal binding sites for small ions are the ionized amino acid side-chains[5].

In order to determine which sites have similar dissociation constants or to compare dissociation constants of a protein to those of smaller model compounds, the electrostatic work of charging the macromolecule must be calculated. Such computations require that the average net protein charge ($\bar{Z}_p$) per molecule be known. If no ions other than the hydrogen ion are bound, $\bar{Z}_p$ is determined by the algebraic sum of the number of positively charged nitrogen atoms and the number of dis-

sociated acid residues and is referred to as $\bar{Z}_H$. Presuming that only univalent anions or cations are bound, the net protein charge is

$$\bar{Z}_p = \bar{Z}_H + \bar{\nu}_{M^+} - \bar{\nu}_{X^-} \quad (1)$$

where $\bar{\nu}_{M^+}$ and $\bar{\nu}_{X^-}$ are the average number of cations and anions bound per molecule of protein respectively. $\bar{Z}_p$ is required for theoretical treatment of the data. This mandates that ancillary data be obtained by alternate techniques to provide estimates of $\bar{\nu}_{M^+}$ and $\bar{\nu}_{X^-}$.

Jespersen and Jordan[6] have shown that the number and type of prototropic groups in ovalbumin could be accurately determined via acid–base thermometric titration by comparing the reaction enthalpies of the various amino acid residues to those of smaller, model compounds. Thermometric titrations show three breaks or slope changes which correspond to the carboxyl, imidazole and amino protons. Methodologically thermometric titration is simpler than studying the effect of temperature on pH and is analogous to the use of spectrophotometric titration for determination of phenolic groups in a protein[7].

The association of small anions and cations to proteins has been investigated by a number of experimental methods including electromotive force (emf) measurements with redox[8] and ion selective electrodes[9], ultra-filtration[10], spectrophotometry[11], osmometry[12,13] light scattering[14] and equilibrium dialysis[15]. It is frequently essential to perform pH studies along with other measurements to obtain $\bar{Z}_p$. The average number of moles of bound X^- per molecule may be estimated by determining the *change* in concentration of the free ion upon addition of the protein. For example[8], $\bar{\nu}_{X^-}$ could be obtained from eqn (2)

$$\bar{\nu}_{X^-} = \frac{m_{X^-} - [X^-]}{m_p} \quad (2)$$

where m_{X^-} is the concentration of free X^- prior to addition of the protein at concentration m_p and $[X^-]$ is the free anion concentration after association. The powerful emf technique cannot be used at high anion concentrations due to the extreme precision required to assess small changes in the concentration of X^-, and this fact is one of the reasons for the present study.

Several workers have shown[16–19] via gravimetry and elemental analysis that the reaction of metaphosphoric acid with proteins is quantitative and that the stoichiometry is dictated by the number of protonated amino acid moieties in the protein.

During the course of the development of a thermometric enthalpy titration for total serum protein[20] we noted that 12-phosphotungstic acid reacted stoichiometrically with proteins even in the presence of a small excess of 12-PTA. This is in accord with the preceding discussion and the general rule, stated by Steinhardt and Reynolds[21], that the intrinsic protein association constant increases with the size of the anion. It is well known that multicomponent mixtures and multi-stage reactions can be examined by thermometric enthalpy titration provided that each component or each stage has a readily measured difference in reaction enthalpy[6]. We were able to

observe that the reaction of 12-PTA with proteins proceeded in two stages because enthalpies of the stages were significantly different. The purpose of this paper is to present our findings on the stoichiometry of these reaction stages for several proteins.

MATERIALS AND METHODS

Proteins and polyelectrolytes

The protein examined most extensively in this work was bovine serum albumin. This material was obtained from Schwarz/Mann and also from Miles Laboratories as the fraction V powder and as the crystallized material. Electrophoresis indicated a minimum protein purity of 97%. No significant difference between the two preparations was observed in this work. Egg albumin (grade V, 1% extraneous protein, crystallized and lyophilized), β-lactoglobulin (crystallized and lyophilized), human γ-globulins (Cohn fraction II, electrophoretic purity 99%) and bovine fibrinogen (fraction IV) were all obtained from Sigma Chemical Co. These materials were not subject to further purification but were stored in a desiccator at 0°C. Stock solutions were prepared by weighing the material into cold distilled deionized water and stirring on a cold-plate stirrer until dissolved. All solutions were stored, refrigerated, and used within two days of preparation. Poly-l-lysine (molecular weight ~139,000) and poly-l-histidine (molecular weight ~6,200) were also obtained from Sigma. Due to the small quantities available these materials were transferred directly to the reaction cell.

Other chemicals

The 12-phosphotungstic acid employed was obtained from Baker. Concentrated stock solutions were prepared by weighing approximately 100 g of the hydrated material and diluting it with distilled deionized water to a final volume of 100 ml. It was occasionally necessary to remove solids by centrifugation. Cesium chloride, employed in this work as a standard, was 99.96% pure and obtained from Fisher. The reagent was dried at 110°C for 24 h and stored in a desiccator. The silver nitrate used was Baker analyzed reagent. It was dried at 110°C for 3 to 4 h and stored in a desiccator in the absence of light. All other chemicals were reagent grade. All solutions were prepared with distilled deionized water obtained from a mixed bed ion-exchange column (Continental Demineralization Service).

Procedure and equipment

The equipment and procedure used to obtain the temperature–volume curves have been described in detail in another paper[20]. A Radiometer Autoburet (Model ABU-II) was used to deliver the titrant; this device has sufficient torque to drive the piston at constant rate against the viscous titrant. Temperature changes were quite small, and this mandated the use of an a.c. Wheatstone thermistor bridge. For some work an Orion digital pH meter (Model 801) and printer (Model 751) were used to acquire a minimum of 20 data points on the rising part of the titration curve. The reaction enthalpies as a function of chloride concentration were estimated by the

initial slope[22] and by the graphical extrapolation method[23]. In preliminary work we noted that the phase lock amplifier, used to measure the temperature changes, was slightly non-linear. This problem was eliminated before enthalpy changes were measured. Enthalpy measurements were made only at low protein concentration in order to avoid the change in viscous heat production upon precipitation of concentrated proteins. All spectrophotometric measurements were obtained on a Cary 14 spectrophotometer, and all protein precipitates were removed by centrifugation on a refrigerated centrifuge (Sorvall, Model RC2-B).

Analysis of supernatant solutions

Some of the data presented here were obtained by determining the change in anion concentrations in the supernatant solution after the protein was precipitated by 12-PTA. For these studies bromide ion was used because it binds to the protein more strongly than chloride and was easier to titrate due to the lower solubility of silver bromide. The amount of bromide in the supernatant was obtained by first removing the protein precipitate by centrifugation. The bromide content of a known volume of clear supernatant was determined via a pre-titration method in which approximately 95% of the bromide was precipitated by adding a weighed volume of silver nitrate standard solution. The remainder of the bromide was titrated with a constant rate syringe pump to a potentiometric end point. Stock bromide solutions containing no protein or 12-PTA could be analyzed with a relative standard deviation of 0.01–0.02%; actual samples titrated with 12-PTA past the second end point (*vide infra*) could be titrated with a precision of 0.05% and samples containing amounts of 12-PTA between the first and second end point could be measured with a precision of 0.1%.

RESULTS AND DISCUSSION

The overall reaction between proteins and 12-PTA

Representative temperature–volume curves for the reaction of a variety of proteins with 12-PTA in 0.1 M hydrochloric acid are shown in Fig. 1. Qualitatively all of the curves are similar in that only two breaks or changes in slope occur. The end points are located by extrapolation from the linear region of each titration curve as noted by the dashed line in Fig. 1. This method of endpoint location is precise even when there is considerable curvature in the vicinity of the endpoint. In order to test for operator bias in visually extrapolating the curves five replicate digital and analog titration curves were recorded simultaneously. The parameters (slope and intercept) of the linear segments of each curve were obtained by least squares fitting. The ratio of endpoint breaks (*vide infra*) was precise to 3–4%, and the average difference between visual extrapolation and least squares fitting was 1.4%[24]. Precipitate formation is observed as soon as the first increment of 12-PTA is added. The temperature–volume curve shows a significant increase in slope at this point. The existence of two slope changes indicates that the process proceeds in at least two distinct stages. The first stage is considerably more exothermic than the second. There are indications in

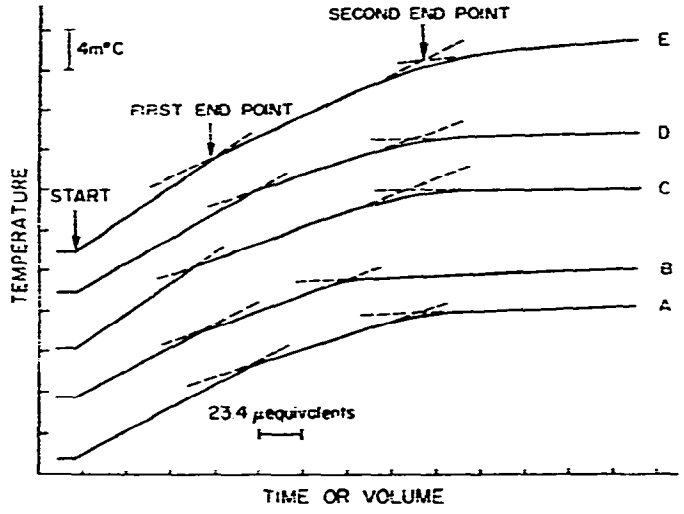

Fig. 1. Representative thermometric titration curves of proteins with 12-phosphotungstic acid. All titrations are carried out at a rate of 2.8 μ equiv. sec^{-1} in 30 ml of 0.1 M hydrochloric acid. The protein type and concentration are: A = 3.00 g l^{-1} ovalbumin; B = 2.00 g l^{-1} B-lactoglobulin; C = 3.70 g l^{-1} bovine fibrinogen; D = 2.00 g l^{-1} bovine serum albumin; E = 3.00 g l^{-1} human γ-globulin.

some of the titration curves of a slight upward or downward slope after the second end point. These are generally encountered in thermometric titrimetry, at the high sensitivity employed in this work, and are due to small unavoidable differences in the temperature of the sample and the titrant. Such differences become more apparent when the measured temperature changes become as small as those involved in this work ($\sim$10 m°C).

Quantitative differences in the stoichiometry of the reaction are summarized in Table 1. The data for stoichiometries are reported as reactive units per 100,000 g of protein in order to avoid any ambiguity due to molecular weight. Literature data are also summarized in this fashion.

Due to the extremely nebulous degree of hydration of the titrant, standardization must be based upon some factor other than weight taken. Results presented elsewhere[20] indicate that 12-PTA reacts quantitatively with cesium salts. By analogy to the documented reaction of cesium with 12-phosphomolybdic acid[25] we believe (*vide infra*) that the stoichiometry of the standardization process is:

$$3Cs^{+} + PTA^{3-} \rightleftarrows Cs_3PTA\ (\downarrow) \qquad (3)$$

All of the data presented in Table 1 and subsequent tables were based on this hypothesis. Thus the *number* of reactive units on a protein was computed assuming that one mole of 12-PTA (three equivalents) reacts with three moles (or equivalents) of cations regardless of their origin.

Our results for the second end point are generally precise to $\pm$0.5% or about $\pm$0.5–1.0 reactive units per molecule of protein provided that its molecular weight is

TABLE I

REACTIVITY OF PROTEINS TOWARD 12-PHOSPHOTUNGSTIC ACID

Protein	*Equivalents of 12-PTA[a] reactive groups/10^5 g*	*Sum of basic and[b] α-amino acids/10^5 g*	*Maximum cationic[c] charge/10^5 g*	*Ratio of breaks[d]*	$\bar{\nu}_{Cl^-}$[e]
Bovine serum albumin	145.2	150.5	147.7[1] (65,000)	0.49	46.2
β-Lactoglobulin	114.2	104.3	112.6[2] (35,500)	0.53	21.5
Ovalbumin	92.0	91.1	91.1[3] (45,000)	0.55	22.8
Human globulin	103.3	99.2	83.7[4] (160,000)	0.58	104
Bovine fibrinogen	91.7	124.9	—[f]	0.63	202[f]

[a] These were determined by calorimetric titration of 30 ml of protein dissolved in 0.1 M hydrochloric acid. The titrant was added at a rate of 2.8 μ equiv. sec^{-1} as determined by titration of cesium chloride under the same conditions. The protein concentrations are in the range 2–5 g l^{-1}. Mean coefficient of variation of all results based on at least three replicates is 0.5%. [b] These data are the sum of the direct amino acid analyses for arginine, lysine, histidine and an estimate of α-amino acids (G. R. Tristram in *The Proteins* by H. Neurath and K. Bailey (eds.), Academic Press, New York, New York, 1953, p. 224). [c] These data are the sum of the basic amino acids as obtained by potentiometric acid-base titration. The number in parenthesis is the molecular weight employed in the reference indicated in the table. (1) C. Tanford, S. A. Swanson and W. S. Shore, *J. Amer. Chem. Soc.*, 77 (1955) 6414; (2) Y. Nozaki, L. G. Bunville and C. Tanford, *J. Amer. Chem. Soc.*, 81 (1959) 5523; (3) R. K. Cannan, A. Kibrick and A. H. Palmer, *Ann. New York Acad. Sci.*, 41 (1941) 243; (4) H. J. Gould, T. J. Gill and P. Doty, *J. Biol. Chem.*, 239 (1964) 2942 -rabbit -globulin. [d] Defined as the ratio of volume in the temperature–volume curve required to go from the first endpoint to the second divided by the total volume. The coefficient of variation is 2.5% based on a minimum of three replicates. [e] This is the average number of moles of bound chloride per molecule of protein calculated on the basis of the ratio of breaks, the indicated molecular weight and the number of reactive groups found. [f] Maximum cationic charge not available. Molecular weight is taken as 350,000 (ref. 24).

in the range of 10^4–10^5 molecular weight units. These data can be compared with two distinctly different measurements. Since the data of Table I were obtained at pH 1, they should be comparable to the maximum cationic charge ($\bar{Z}_{H,max}$) as obtained from acid–base titration. Secondly, the positive charge sites are the basic amino acids plus N-terminal α-amino acids. Thus the data can be compared to direct amino acid analysis. In most cases the agreement is acceptable. This indicates that our assumption concerning reaction (3) is valid. Discrepancies do occur with fibrinogen; the material employed was not very pure, and the error is related to the presence of unreactive material. Nevertheless, fibrinogen has the highest molecular weight (~350,000) of all of the proteins tested and exists as a rod shaped molecule with three compact areas[26] which may not be completely accessible to the titrant. A second complication in comparing the data is that at pH 1 the proteins may have uncoiled to a greater extent than in the acid–base titration studies which are generally terminated at pH 2. This may permit the reaction of additional sites which are buried on the interior of the protein. In general the agreement indicates that the *overall* reaction stoichiometry is:

$$H_nP^{n+} + \frac{n}{3}PTA^{3-} \rightleftarrows H_nP(PTA)_{\frac{n}{3}}(\downarrow) \qquad (4)$$

where H_nP^{n+} is a *fully protonated* protein, and n is equal to the maximum cationic charge ($\bar{Z}_{n,max}$). The reaction of 12-PTA with a protein can be used as a relatively simple check on the direct amino acid analysis of proteins which avoids the computational and measurement difficulties of the acid–base titration. It does not provide nearly as much information about the prototropic groups in the protein as does acid–base titration with a glass electrode or thermometric acid–base titration; however, a large discrepancy should provide some insight into the availability of the basic residues for protonation. The absolute requirement of the reaction for a positive charge on the protein was demonstrated by studying the effect of an increase in pH on the equivalency. The data are summarized in Table 2, and representative titration

TABLE 2
EFFECT OF pH ON THE NUMBER OF REACTIVE GROUPS[a]

pH	*Buffer*[b]	*Equivalents/100,000 g*	*Ratio of breaks (R)*
0.	1.0 M HCl	146.1[c]	0.74[d]
1.0	0.1 M HCl	145.2[c]	0.49[d]
2.0	0.240 M H_3PO_4	141.6[c]	0.47[d]
3.0	0.220 M citric acid	128.2[c]	0.45[d]
4.0	0.656 M acetic acid	104.4[c]	—[e]
5.0	0.121 M acetic acid	76.5[c]	—[e]
6.0	0.137 M H_3PO_4	no break	—[e]

[a] Protein concentration 2.00 g l^{-1} of bovine serum albumin; 12-PTA concentration 0.648 N, volume rate of addition 2.18 μl sec^{-1}. [b] The buffers were brought to the indicated pH with sodium hydroxide. [c] These are the mean of duplicate determinations, average range of 0.5%. [d] Distance from first to second break divided by the total volume. Average range of duplicate determination is 3-4%. [e] These curves were too ill-defined to measure the ratio of breaks with precision. The final end point could be located with a precision of approximately 2%.

curves are shown in Fig. 2. It is obvious that pH has a drastic effect on both the shape of the curves and the quantitative results obtained from them. Due to the ill-defined shape of the titration curve above pH 2–3 the precision of end point location deteriorates considerably. The titration curves at pH 4 and 5 are included only for the sake of completion; we are not convinced that there are two breaks in this pH region and no significant quantitative results should be inferred from them. The highly exothermic slopes of the post titration branch are due to decomposition of the titrant above pH 3.

Effect of pH on the reaction stoichiometry

The equivalency per 100,000 g of protein generally decreases with increasing pH. Our results adjusted to a molecular weight of 65,000 are compared to those of Tanford[7] in Fig. 3. If the number of equivalents (h) of dissociated hydrogen ion per mole of protein is subtracted from the maximum cationic charge ($\bar{Z}_{H,max}$), the net *proton* charge, $\bar{Z}_H$, is obtained:

$$\bar{Z}_H = (\bar{Z}_H)_{max} - h \tag{5}$$

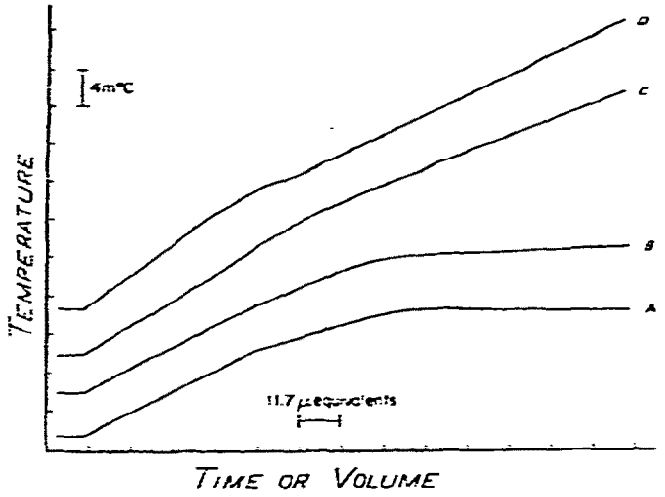

Fig. 2. Dependence of the thermometric titration curve on pH. All solutions contain 2.00 g l^{-1} bovine serum albumin in 30 ml of buffer; titrant added at a rate of 1.4 μ equiv. sec^{-1}. The solution pH are as follows: A = pH 2.0 (0.24 M phosphate); B = pH 3.0 (0.22 M citrate); C = pH 4.0 (0.66 M acetate); D = pH 5.0 (0.12 M acetate).

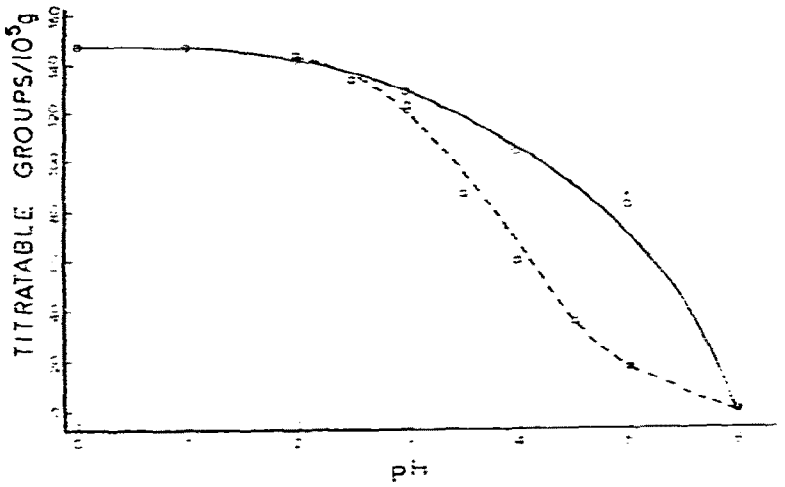

Fig. 3. Effect of pH on the end point stoichiometry for the reaction of bovine serum albumin with 12-phosphotungstic acid. ○ = equiv./10^5 g as determined in this work; □ = proton charge/10^5 g (see ref. 7).

Since the first prototropic groups to ionize are the carboxylic acids, the initial decrease in $\bar{Z}_H$ and in the precipitation stoichiometry is due to the accumulation of negative sites. Figure 3 indicates that our results are in good agreement with acid–base titration data up to pH 3. Beyond this pH our results are too high. This may be due, in entirety, to dissociation and rearrangement of the 12-PTA as the pH increases and

to the ill-defined titration curves. We have noted that the reagent is unstable in weak acid or neutral media, and that when the titrant is preadjusted to pH 3 it *does not precipitate cesium salts*. Others indicate that proton transfer reactions of 12-PTA are time-dependent above pH 3 (ref. 27). Nonetheless, cesium in a pH 3 buffer will be precipitated provided that the reagent is *not* preadjusted or neutralized; however, the equivalency is very much decreased. For this reason all of our results are reported with the titrant standardized against cesium in 0.1 M hydrochloric acid. We conclude that the equivalency of 12-PTA at the end point is fixed by $\bar{Z}_H$ provided that the titrant is used under conditions where decomposition is minimal.

Effect of protein concentration on reaction stoichiometry

The data presented thus far pertains to proteins in an optimum concentration range. It appears that outside the limits 1–10 gl^{-1} of bovine serum albumin the stoichiometry tends to increase. This is illustrated by the data of Table 3. We have shown[20] that at low concentration the increase in stoichiometry is due to a decrease in the net reaction rate which produces a spuriously prolonged end point[28]. At high protein concentrations the viscosity change upon protein precipitation is so significant that the end point is drawn out and somewhat obscured[20].

TABLE 3

EFFECT OF BOVINE SERUM ALBUMIN CONCENTRATION ON THE RATIO OF BREAKS

Protein conc. ($g\ l^{-1}$)	*Equivalents/ 100,000 g*[a]	*Ratio of breaks*[b]	$v_{Cl}^{-b,d}$	*Ratio of breaks*[c]	v_{Cl}^{-d}
0.50	164.4	0.52	50.5		
1.00	146.6	0.52	50.5		
2.00	145.2	0.50	48.5	0.65	63.2
5.00	144.0	0.43	41.6		
10.00	145.9	0.38	36.8		
20.00	154.6	0.32	31.0	0.67	65.0

[a] For titrations carried out in 30 ml of 0.1 M hydrochloric acid, the mean coefficient of variation for three replicates over the range of concentration 1.0–10.0 g l^{-1} is 0.8%. All other conditions as in Table 1. [b] This number is calculated as in Table 1. The coefficient of variation based on three replicates is 6–8%. The data is obtained in 0.1 M hydrochloric acid. [c] These data were obtained in 0.5 M hydrochloric acid. The standard deviation of four replicates at each concentration is ±0.02. [d] Calculated as the product of ratio of breaks and maximum cation charge per molecule.

The intermediate end point region

The first end point in Fig. 1 was initially believed to be a result of enthalpy differences between the various types of amino acids as found by Jespersen and Jordan for the thermometric acid–base titration of proteins. In order to test this hypothesis two poly-peptides (poly-l-histidine and poly-l-arginine) were titrated. Both peptides contain only one type of amino acid, yet two breaks were observed in

each case. Thus the first break in the titration of a protein must be due to some factor other than the presence of a variety of amino acids.

In order to determine the nature of the first break, a titration of 10 g l^{-1} of bovine serum albumin in 0.1 M hydrochloric acid with 0.34 N PTA was carried out. The titration was stopped abruptly after the appearance of the first break but well before the second break. The contents of the reaction vessel were centrifuged and the solids removed. The supernatant fluid contained less than 1% of the amount of protein expected on the basis of the second end point. Subsequent titration of the supernatant solution gave no further precipitate or heat. The protein precipitate was then titrated and a *single* well-defined break was observed at a volume close to the volume difference between the first and second titration end points.

Three samples of BSA at a concentration of 2 g l^{-1} in 0.1 M hydrochloric acid were titrated. One titration was stopped before the first end point; upon vigorous stirring the precipitate dissolved completely within 15 min. The second and third titrations were stopped before and after the second end points, respectively; the precipitate did not dissolve even after three days. Clearly, there is a qualitative difference in the nature of the precipitate formed prior to and after the first end point and that the initial material is not thermodynamically stable. For most of the results reported here the titrations were completed within 2 min and in general the first end point was obtained within 1 min. Thus on the time scale of these titrations the extent of redissolution of the precipitate was small.

We were not able to add the 12-PTA during a titration and disperse the reagent so rapidly as to prevent precipitation. However, several experiments were conducted in which a small volume of 2 g l^{-1} of BSA was dialyzed against a large volume of 12-PTA at concentrations of $\sim 2 \times 10^{-5}$ and 2×10^{-4} M in 0.1 M hydrochloric acid; after three days of dialysis at 10°C no precipitate was observed. Addition of a reducing agent indicated that 12-PTA was present in the protein solution. Direct addition of dilute (2×10^{-5} M) 12-PTA to the dialyzed protein immediately produced a precipitate. We conclude that the initial formation of precipitate, under titration conditions, is due to local excesses of the titrant. The precipitate is kinetically stable, i.e., slow to redissolve.

In order to assess the extent of removal of protein prior to the first end point, the experiments whose results are summarized in Fig. 4 (curves a and b) were carried out. These measurements were conducted by addition of the indicated volume of 12-PTA to a series of samples of acidified BSA. The resultant solutions were centrifuged immediately at 5000 rpm in a refrigerated centrifuge at 10°C. The separations were completed within 5 min. The solids were air-dried at room temperature and weighed; the supernatants were analyzed for protein by biuret colorimetry. Comparison of curves a and b with curve d of Fig. 5 indicate *complete removal of the protein* slightly past the first end point but well *before the second thermometric end point*. The scatter, in the results is probably due to some redissolution of the material during centrifugation. The near coincidence of the intersection points of curves a and b

with the first end point of curve d indicates that redissolution in the shorter time of the thermometric titration is probably negligible.

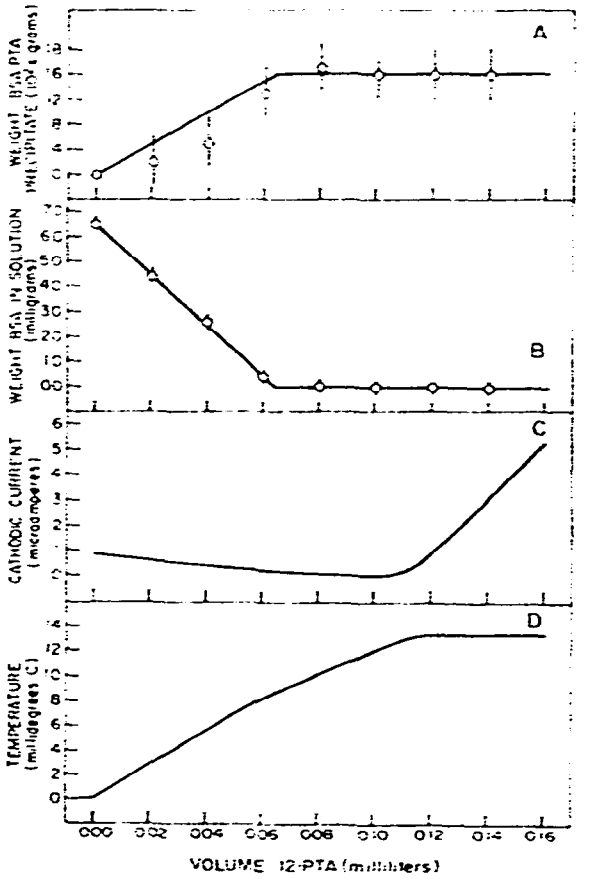

Fig. 4. Variation in the concentration of bovine serum albumin and 12-phosphotungstic acid during titration. All curves are for 2.0 g l^{-1} protein in 30 ml of 0.01 M hydrochloric acid. A = the increase in weight of air dried precipitate obtained by centrifugation; B = decrease in protein concentration in supernatant as measured by biuret colorimetry; C = reduction current of 12-phosphotungstic acid at −0.25 V versus saturated calomel electrode; D = thermometric titration curve.

Since 12-PTA is electroactive[29], we were able to monitor its concentration directly during a constant rate titration through the use of a rotating gold electrode. The details of this procedure have been published elsewhere[30]. It is evident from curve c of Fig. 6 that very little 12-PTA accumulates in the solution prior to the second end point. The slight decrease in current seen in this figure is due to adsorption of the protein on the solid micro electrode.

From the above results we conclude that essentially all of the protein is precipitated from solution at the first end point in the titration curve and that the precipitate reacts with additional titrant to form the final product. In order to preserve

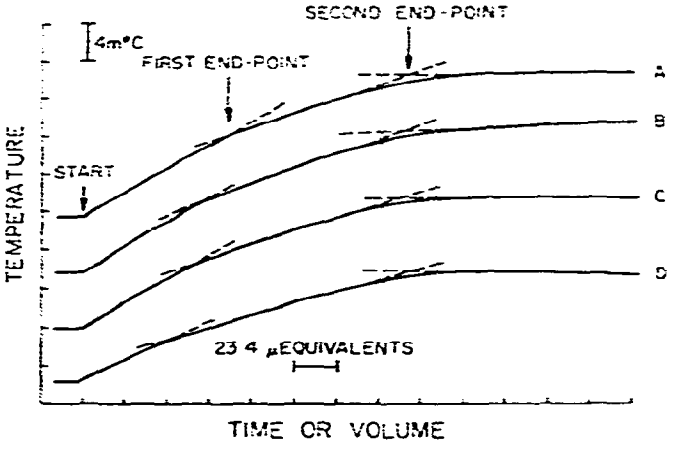

Fig. 5. Effect of sodium chloride on the thermometric titration curve of 2.0 g l^{-1} bovine serum albumin. All solutions contain 0.10 M hydrochloric acid to which is added the following concentration of sodium chloride: A = 0.10 M; B = 0.30 M; C = 0.50 M; D = 0.90 M.

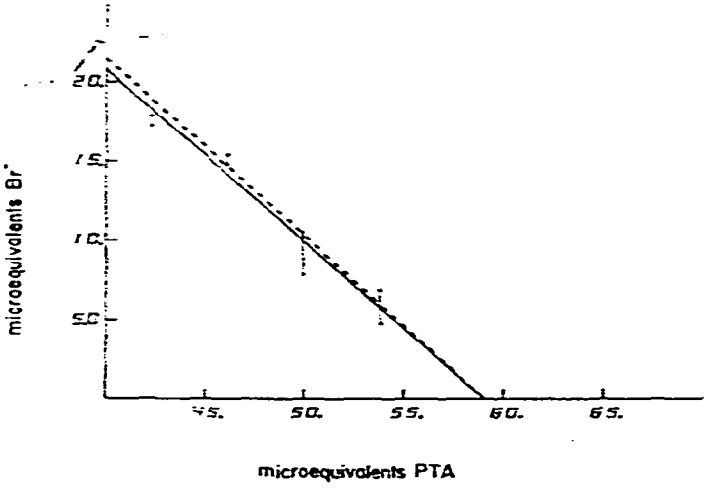

Fig. 6. Correlation between microequivalents of bromide found by analysis and that predicted by the amount of 12-PTA added. Titrant added at a rate of 0.38 μ equiv. sec^{-1} into 20 ml of 3.3 g l^{-1} of protein in 0.3 M HBr. Dashed line is theoretical result. Solid line is linear least squares regression. Bars represent the range of duplicate determinations.

electroneutrality of both the precipitate phase and the solution, it is necessary to assume that either some cation is removed from the protein or an anion other than 12-PTA is present on the precipitated protein at least up to the second end point. It is unreasonable to assume the loss of H^+ from the protein at pH 1; therefore, the observations suggest the sequence of reactions given below:

Before titration:

$$P + nH^+ + \nu_{Cl^-} \rightleftarrows H_nP(Cl)_\nu^{(n-\nu)+} \quad (6)$$

Before first end point:

$$H_nP(Cl)_\nu^{(n-\nu)+} + \frac{n-\nu}{3} PTA^{3-} \rightleftarrows H_nP(Cl)_\nu(PTA)_{\frac{n-\nu}{3}} (\downarrow) \quad (7)$$

After first end point:

$$H_nP(Cl)_\nu(PTA)_{\frac{n-\nu}{3}} + \frac{\nu}{3} PTA^{3-} \rightleftarrows H_nP(PTA)_{\frac{n}{3}} (\downarrow) + \nu_{Cl^-} \quad (8)$$

Reaction (6) represents the complete protonation of the protein and the binding or condensation of chloride ions on some of the positive charge sites. Reaction (7) proceeds after the start of the titration up to the first break and describes the precipitation of the protein by 12-PTA *without* displacement of condensed chloride ions. After the first end point reaction (8) dominates and addition of 12-PTA forces chloride ion displacement from the protein. It should be noted that we are assuming that the reaction sequence is completed so rapidly that at most only a small fraction of the first precipitate redissolves. Further the model assumes that in reaction (7) no chloride is displaced by the titrant. This is likely in that very little 12-PTA and a large excess of chloride is present in the reaction system but the assumption is not essential to the model.

This model is qualitatively consistent with the existence of two end points in the titration of both the proteins and the polypeptides tested, the absence of 12-PTA before the second end point, the complete removal of protein at the first end point, the reactivity of the initial precipitate and the variation in the ratio of breaks as a function of pH, buffer, anion type and chloride concentration. Changes in this ratio

TABLE 4

EFFECT OF SODIUM CHLORIDE ON THE NUMBER OF REACTION GROUPS[a]

[NaCl], M	*Equivalents/ 100,000 g*[b]	*Ratio of breaks*[c]	ν_{Cl^-}[d]	Z_p[e]
0	145.2	0.49	47.5	49.5
0.10	145.8	0.54	52.5	44.5
0.30	146.6	0.62	60.1	36.9
0.50	144.1	0.64	62.1	34.9
0.90	145.4	0.72	69.9	27.1

[a] All titrations were carried out in 0.1 M hydrochloric acid with the addition of the indicated amount of sodium chloride. The protein is BSA at a concentration of 2.00 g l^{-1}. Other conditions as in Table 1. [b] Mean coefficient of variation of a minimum of three replicates at each concentration is ±0.4%. [c] Mean coefficient of variation based on three replicates is 3%. [d] Calculated from the ratio of breaks and maximum cationic charge per molecule. [e] $Z_p = Z_{H,max.} - \nu_{Cl^-}$.

are evident in the data of Tables 2 and 4 and in the titration curves shown in Figs. 2 and 5. Furthermore the trends are consistent with the data of Tanford[7] and with the results of Scatchard and Yap[8].

Effect of chloride ion on the intermediate end point

The data of Table 4 and Fig. 5 show that the concentration of chloride ion has a very pronounced effect on the ratio of breaks. Presuming that the scheme given by reactions (6)–(8) is correct, the number of moles of bound chloride per mole of bovine serum albumin *at pH less than 2* should be given by:

$$\bar{v}_{Cl^-} = R \cdot \bar{Z}_{H,max.} \tag{9}$$

Furthermore, some chloride should be found on the protein precipitate. This proved to be the case; however, we could not quantitatively measure the chloride due to occlusion of supernatant solution in the precipitate. The actual amount of firmly bound anion can be measured by separating the protein precipitate from the supernatant solution and determining the change in anion concentration similar to the method based on eqn (2). For the data given in Figs. 6 and 7 measurement of the change in anion concentration proved to be difficult due to the small relative changes which are always less than 5% and frequently less than 1%.

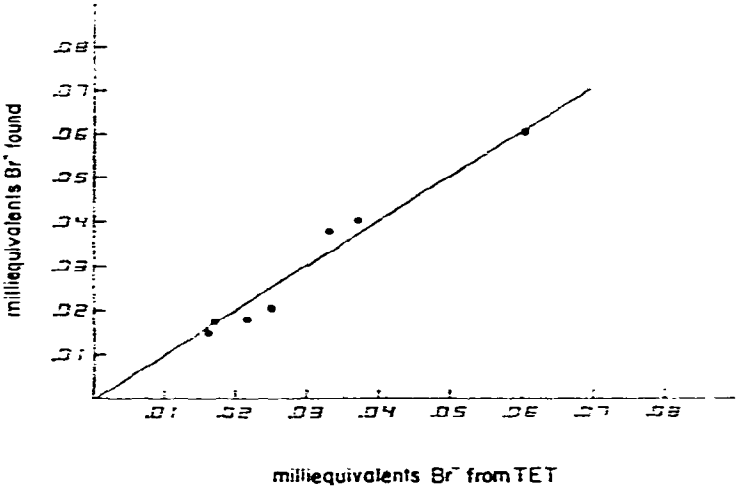

Fig. 7. Correlation between microequivalents of bromide found by analysis and the amount predicted by the ratio of breaks (eqn (9)). Solid line is the theoretical correlation, points are experimental data for various protein (3–4 g l^{-1}), and bromide (3–200 mM) concentrations.

The data of Fig. 6 represent the change in moles of bromide bound by the protein as determined by analysis of the supernatant as a function of equivalents of 12-PTA added. The plot should have a slope of -1 (actual $= -1.09 \pm 0.11$) and should intersect the X-axis at 0.0594 (actual $= +0.059 \pm 0.010$) based upon a thermometric titration with 12-PTA.

A study similar to that of Fig. 6 was carried out as a *function of the bromide concentration of the supernatant solution* (see Fig. 7). Thermometric titrations of BSA were carried out to a point definitely past the first end point, the titration stopped and the extent of bromide binding determined by supernatant analysis. The amount of bromide bound to the precipitate at that point on the curve versus the amount predicted by the thermometric titration is given in Fig. 7. This line should have a unity slope (actual = 1.04 ± 0.05) and intercept of zero (actual = -0.001 ± 0.002).

The above data indicate that the reactions (7) and (8) proposed to explain the existence of two breaks in the titration curve are essentially correct; however, this does not mean that we are measuring all bound anions by the procedure since there may be very weakly bound ions which are readily displaced. We have found that the ratio of breaks is rather independent of the rate of titrant addition. In order to determine whether the technique is measuring all bound anions, the data were plotted in Fig. 8 according to the approach developed by Scatchard using the Linderstrom-Lang model for electrostatic corrections. We assumed the existence of two classes of sites. The first class contains 27 completely saturated sites and a second set of sites which fills as the chloride concentration increases. In order to correct the data for electrostatic charge effects we assumed a radius of 50 Å for the protein which is in good agreement with electrophoretic, sedimentation and partial specific volume

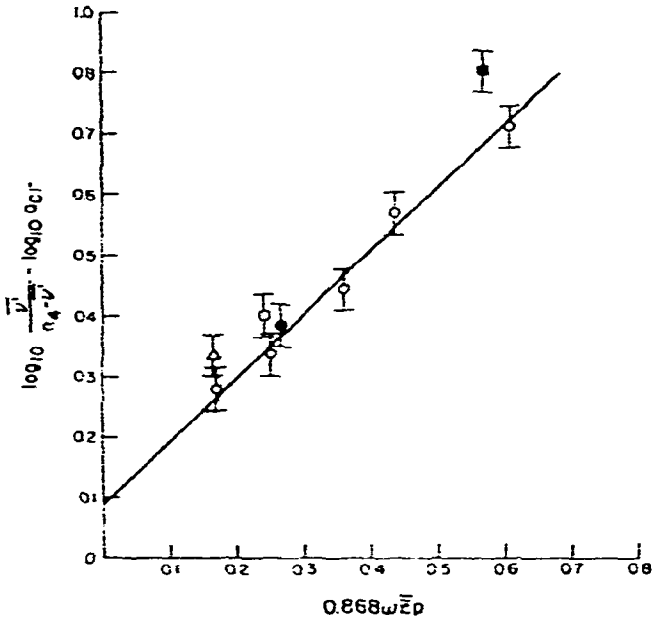

Fig. 8. Analysis of chloride binding to bovine serum albumin (see Table 3). The data were plotted according to the approach developed by Scatchard. The radius of the protein is taken as 50 Å, $n_4 = 70$. ○, 2.00 g l^{-1} protein in 0.10 M hydrochloric acid containing added sodium chloride; ●, 2.00 g l^{-1} protein in 0.50 M hydrochloric acid; □ 20.0 g l^{-1} protein in 0.50 M hydrochloric acid; △, 2.00 g l^{-1} protein in 1.0 M hydrochloric acid; ■, 0.5 and 1.0 g l^{-1} protein in 0.10 M hydrochloric acid no sodium chloride added.

studies on BSA in very acidic solution[31,32]. The activity coefficient of chloride was estimated from Scatchard's empirical equation and in accord with his work we have neglected the protein's contribution to the ionic strength. As the data of Fig. 8 indicate our measurements correlate reasonably well with the equations of ion binding and have the expected slope of unity (actual = 1.04±0.07). The intercept of Fig. 8 indicates an intrinsic binding constant of 1.1–1.4 for the unsaturated class which contains a total of 65–70 individual sites. The total number of chloride binding sites is 92–97 per molecule indicating that virtually all of the protonated amino acids can serve to bind anions. This is in agreement with Scatchard's work in very concentrated acidified sodium chloride.

As yet, we are at a loss to explain the decrease in the ratio of breaks at high protein concentrations. This could be explained by dimerization of BSA, which has been reported by others[33], but may be due to the protein's contribution to the ionic strength. As noted in Talbe 3 this effect is severely attenuated in concentrated chloride media.

Reaction enthalpies

Although there is considerable curvature evident in virtually all of the titrations shown here, the two breaks are not spurious artifacts of the methodology. This is strongly supported by the enthalpy data reported in Table 5. These data, obtained by both the graphical extrapolation method[23] and the initial slope method[22], were always in good internal agreement (±5%). The enthalpies are reported as kcal mol^{-1} of reactive site, not per mole of 12-PTA. It is evident that chloride concentration has a very strong influence on the ratio of breaks (see Table 4). The data of Table 5 indicate that the apparent enthalpy of each segment of the titration curve is essentially independent of chloride concentration as one expects to be the case for a chemical reaction. Most significantly the enthalpy (in kcal mol^{-1}) of each segment of the titration curve is independent of the amount of protein titrated. We believe that this

TABLE 5

EFFECT OF PROTEIN AND CHLORIDE CONCENTRATION ON APPARENT REACTION ENTHALPY

Protein conc. ($g\ l^{-1}$)	*Chloride conc.* (M)	$-\Delta H_1$[a] ($kcal\ mol^{-1}$)	$-\Delta H_2$ ($kcal\ mol^{-1}$)
1.67	0.10	6.5±0.3	3.7±0.1
1.67	0.30	6.2±0.4	3.2±0.2
1.67	0.50	6.3±0.4	3.2±0.1
1.67	1.00	5.1±0.3	3.0±0.1
0.83	0.10	6.4±0.5	3.7±0.4
3.33	0.10	6.9±0.2	4.0±0.4
6.67	0.10	6.4±0.3	4.2±0.3
10.0	0.10	6.1±0.3	3.6±0.3

[a] Heats are per mole of reactive unit in the protein; indicated precision is the standard deviation based on a minimum of three replicates. All enthalpies are exothermic.

would be a most unlikely result if here were not two distinct stages of reaction each of which corresponds to a well-defined stoichiometric reaction.

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