

Reversible Denaturation of Human Serum Albumin by pH, Temperature, and Guanidine Hydrochloride Followed by Optical Rotation

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SUMMARY

The pH-dependent transitions and the thermal unfolding of defatted human serum albumin in 0.2 M KCl as followed by polarimetry at 233 nm are found to be independent processes. The apparent net loss of structure (α helix) in the unfolding between pH 7 and 9 is 2.5%, in the N-F transformation it is 8% and in the acid expansion 1%. The two last mentioned transitions seem to be independent of each other. There is a temperature of maximum stability of the N stage in the N-F transformation at 18°.

The thermal unfolding and the unfolding by guanidine hydrochloride at pH 2.6 both are continuous processes of apparently low cooperativity with no distinct intermediates. The temperature of maximum stability (T_{max}) of human serum albumin is at all pH values below 0°, furthermore it is found to vary continuously with the concentration of guanidine hydrochloride, and in 0.2 M KCl at pH 2.6, T_{max} can be extrapolated to -6°.

From the transition profiles for thermal denaturation, the three dimensional structure of human serum albumin might be pictured to consist of segments of helical configuration with differential stabilities toward denaturation which impart the ability to unfold simultaneously, but more or less independently.

An extensive body of experimental data documenting the pH-dependent transitions of human serum albumin is available (1-5), but surprisingly few investigations have been performed to characterize their temperature dependence. The electrophoretic mobility of bovine serum albumin has been determined (6), and titration curves have been obtained (7) at two different temperatures. However, no such data are available for human serum albumin. The acid transformations and the transition between pH 7 and 9 have been followed by polarimetry at 313 nm for both HSA¹ (2, 3) and bovine serum albumin (2, 3, 8), but only

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¹ The abbreviations used are: HSA, human serum albumin; BSA, bovine serum albumin; OR, optical rotation.

for BSA at 233 nm (9). No data on the optical rotation of the reversible thermal denaturation or unfolding by guanidine hydrochloride of neither BSA nor HSA has been reported.

The aim of this investigation is to characterize the pH-dependent conformational changes of defatted HSA at different temperatures, to determine the thermal transition profile at different pH values and concentrations of guanidine HCl, and finally to investigate the unfolding induced by guanidine HCl as a function of pH by monitoring the accompanying polarimetry changes at 233 nm.

The experimental approach and the analysis of the data follow the lines summarized recently by Tanford (10, 11).

METHODS

Human serum albumin was obtained from Behringwerke (Lot 3142). The protein is prepared by Rivanol ammonium sulfate precipitation, immunochemical purity 100%. The dimer content was 6% as determined by polyacrylamide electrophoresis. The HSA was defatted by charcoal treatment at pH 2.75 (12) and deionized on a mixed bed deionizing column (13). The free -SH groups were blocked with iodoacetamide (Sigma), 5 moles per mole of protein at pH 7 for 24 hours, and the protein was dialyzed extensively against 0.2 M KCl. In all steps following the defatting procedure, the salt concentration was kept at 0.2 M KCl (9). All solutions were filtered on rinsed Millipore filters (SXHA 025, OS 0.45 μ). The protein solutions were stored at 4°. No change in specific rotation at 233 nm could be measured up to 8 weeks after the preparation.

Protein concentrations were determined by measuring optical density at 280 nm ($E_{1\%}^{1\text{cm}}$ 5.30) on a double beam spectrophotometer (Cary 14). The concentrations of the stock solutions were controlled by dry weight determinations.

The OR measurements were made on a Cary 60 spectropolarimeter with thermostated cell holder. The temperature in the cell holder was recorded continuously by a thermistor and from the measurements the temperature in the center of the cell was obtained by means of a calibration curve. In all experiments a 1- or 10-mm cuvette was used.

The samples were adjusted to the desired pH value with 0.1 or 0.5 M HCl or NaOH solutions while thermostated at the temperature of the OR measurement. The pH meter (Radiometer TTT 1a) was calibrated at each temperature. The pH of the

samples was found to be within 0.1 pH unit of the adjusted value when checked after the OR measurements. All experiments were performed in 0.2 M KCl.

Guanidine HCl was purified according to the method of Reithel and Sakura (14) by recrystallization from methanol. The molar extinction at 233 nm was less than $0.02 \text{ M}^{-1} \text{ cm}^{-1}$.

The samples with guanidine HCl were prepared in 3-ml volumetric flasks, the solutions were adjusted to the pH wanted and then transferred to the flasks by constriction pipettes. For concentrations higher than 6 M, the crystalline guanidine HCl was weighed directly in the volumetric flasks.

In most experiments the rotation of the sample was followed solely at 233 nm, however, a few control scannings were performed from 350 to 225 nm. Dispersion data were analyzed using the Shechter-Blout equation (15, 16) and assuming a mean residue weight of 115 g mole^{-1} (17).

The specific rotations at 233 nm were corrected for the refractive indices of water and of guanidine HCl, and expressed as the mean residue rotation $(m')_{233}$. The refractive indices at the sodium D line for different molarities of guanidine HCl were obtained from Dr. Tom Hooker.² The variation of the refractive index of solutions of guanidine HCl with wave length and temperature is unknown, thus the index at the sodium D line was used without correction.

The helix content, fh , was calculated from the mean residue rotations at 233 nm according to Simmons *et al.*³ (18):

$$fh = \frac{[m']_{233} \text{ folded protein} - [m']_{233} \text{ unfolded protein}}{-12700}$$

The reference values for 100% ($-14,600$) and 0% ($-1,900$) helix were those deduced for poly(L-glutamic acid) (19). The $(m')_{233}$ for the totally unfolded HSA was obtained from the data in 8 M guanidine HCl (Figs. 6 and 7).

The equilibrium constant for a particular unfolding process is calculated as

$$K = \frac{fd}{1-fd}$$

where fd is the fraction denatured determined as

$$fd = \frac{[m']_{233} - n}{d - n}$$

n is the mean residue rotation of the folded state, d the rotation of the unfolded, and $(m')_{233}$ the mean residue rotation at the particular experimental conditions (10).

RESULTS AND DISCUSSIONS

All the observed unfolding reactions of HSA in 0.2 M KCl were found to be completely reversible for temperatures up to 50° and at pH 2.6 even to 60° . In 2 to 3 M guanidine HCl the reactions were irreversible at temperatures higher than 30° , but at higher molarities the process was again reversible up to 50° within the experimental time of 8 hours. The reversibility of the unfolding of HSA from various concentrations of guanidine HCl was checked either by dilution or by gel filtration of the samples from a given molarity of guanidine HCl to 0.2 M KCl.

The hydrogen ion-dependent transition between pH 7 and 9

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³ The calculations are regarded as a convenient measure of structure content more than a precise determination of the amount of α helix.

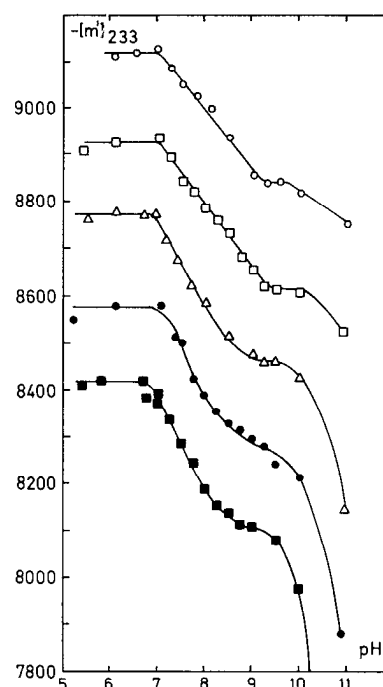


FIG. 1. The mean residue rotation at 233 nm of defatted HSA as a function of pH from 5.5 to 11 at the temperatures: \circ — \circ , 11° ; \square — \square , 20° ; \triangle — \triangle , 30° ; \bullet — \bullet , 37.5° ; \blacksquare — \blacksquare , 44° . KCl, 0.2 M; protein concentration around 0.05%; 1 cm cell.

TABLE I
pH-dependent transitions of defatted HSA at different temperatures

Temperature	Unfolding between pH 7 and 9 (the N-B transition)			The N-F transition			ΔH_{appNF} (from Fig. 4) Cal/mole
	pH of midpoint	$\Delta \nu_{\text{H}^+}$	r value ^a for $\Delta \nu_{\text{H}^+}$	pH of midpoint	$\Delta \nu_{\text{H}^+}$	r value ^a for $\Delta \nu_{\text{H}^+}$	
11°	8.19	-0.894	0.986	4.24	2.67	0.994	-2.4
20	8.09	-0.824	0.986	4.24	2.58	0.996	0.8
30	7.83	-0.984	0.994	4.28	2.57	0.997	9.2
37.5	7.83	-1.076	0.983	4.39	2.58	0.998	23
44	7.66	-1.145	0.999	4.57	2.43	0.998	38

^a The Bravais Pearson coefficient of linear correlation.

(Fig. 1) of defatted HSA at temperatures from 11 – 44° is coincident with the one determined by polarimetry at 313 nm by Leonard *et al.* (3). The unfolding will here be designated the N-B transition where B is the stage before the large steep transition at pH above 10. The optical rotation of both the N and the B stage is temperature-dependent. The midpoint of the N-B transition is displaced toward neutral pH by elevating the temperature (Table I). The steepness of the transition is expressed as

$$\frac{\partial \ln K}{\partial \ln a_{\text{H}^+}}$$

which is equal to the preferential binding of protons $\Delta \nu_{\text{H}^+}$ in the transition, if the unfolding is a two-stage process (20). The pH-dependent unfoldings of serum albumin are usually regarded to be cooperative processes⁴ (1). However, the experimentally

⁴ Cooperativity in relation to globular proteins has according to Tanford (10) the experimental consequence that major changes

TABLE II

Helix content of HSA at different pH and temperatures calculated on basis of mean residue rotation at 233 nm (m'_{233}) (18)

pH	Temperature	HSA, ^a 0.2 M KCl	BSA, ^b 0.1 M KCl
		%	%
5.6 (N stage)	20°	51.8 (56.0)	51.2
3.4 (F stage)	20	41.5 (46.4)	43.6
2.6 (E stage)	20	40.7 (45.5)	34.6
9.4 (B stage)	20	48.8	
5.6	6	58.4	
5.6	50	44.6	
2.6	2	45.8	
2.6	50	26.8	
7.5	37.5	46.7	

^a The values in parentheses are the ones deduced with non-helical poly(L-glutamic acid) as (m'_{233}) for completely unfolded HSA.

^b From Sogami and Foster (9). These authors have used the same reference values of (m'_{233}) for 100% and 0% of helical content (19) as in this paper but have presumably used the value of (m'_{233}) for nonhelical poly(L-glutamic acid) as the value for the completely unfolded BSA. The value for completely unfolded HSA used in the present calculations are the one in 8 M guanidine HCl determined with regard to its temperature (Fig. 7) and pH (Fig. 6) dependence.

observed transitions is usually not sufficiently steep to be compatible with a two-state process (21). This has been explained by the microheterogeneity of the serum albumin preparations, the subspecies being differently stable toward the denaturation (22, 23). An appreciable part of the heterogeneity of BSA has been demonstrated to be caused by fatty acids bound to the albumin (9).

The apparent preferential binding of protons in the N-B transition of defatted HSA, calculated from the equilibrium constants (see "Methods"), is around -1 and increases with temperature (Table I). From titration curves on nondefatted BSA it has recently been demonstrated that the N-B transition is provoked by the titration of several histidyl groups with abnormal high pK values (8). The N-B transition of nondefatted HSA is similar to that of nondefatted BSA when measured by polarimetry at 313 nm (3). From the present determination of $\Delta\nu_{H^+appNB}$ it can therefore be concluded that the N-B transition of defatted HSA in KCl solutions is still not a two-state process when measured by polarimetry and the equilibrium constant determined in the transition must be apparent.

The amount of "helical" structure involved in the N-B transition is small (Table II) and is almost unchanged by temperature. This is more clearly demonstrated in Fig. 2, where shifts in pH between 6.5 and 9.0 are seen to cause only parallel displacements in the variation of OR with temperature. Thus, it can be directly concluded from Figs. 1 and 2 that the N-B transition and the thermal unfolding of HSA in the neutral and slightly basic pH range are virtually independent processes.

The hydrogen ion-dependent transitions in the acid pH range (Fig. 3) can be divided without difficulty into two regions, the exact limits being dependent on temperature. (a) The sigmoidal transition (pH 3.8 to 5.0 with midpoint at pH 4.2 at 20°)

in conformation, involving many discrete chemical events, is provoked by small changes in temperature or concentration of denaturing agent, *i.e.* the transformation from one state to another is 'steep'.

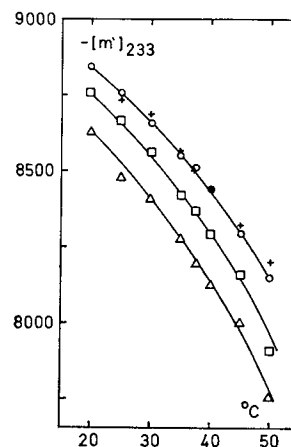


FIG. 2. The mean residue rotation at 233 nm of defatted HSA in variation of temperature at different pH values: +—+, pH 6.5; O—O, pH 7.0; □—□, pH 8.0; △—△, pH 9.0. KCl, 0.2 M; protein concentration around 0.05%; 1 cm cell. Each experimental point represents a separately prepared sample.

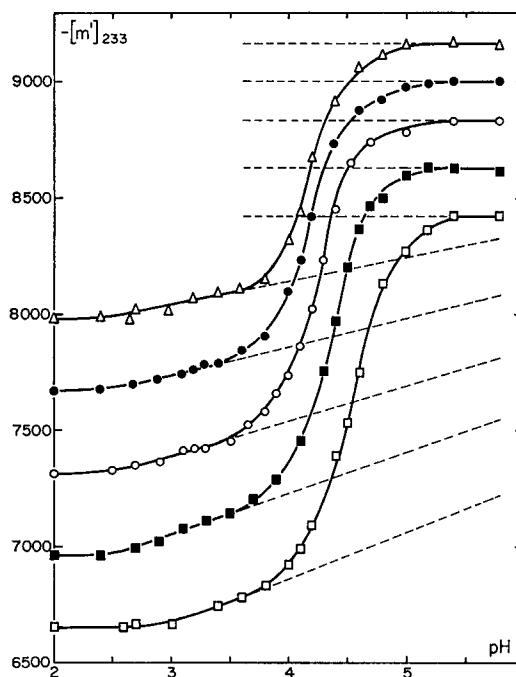


FIG. 3. The mean residue rotation at 233 nm (m'_{233}) of defatted HSA as a function of pH from 5.5 to 2.0 at the temperatures: △—△, 11°; ●—●, 20°; ○—○, 30°; ■—■, 37.5°; □—□, 44°. KCl, 0.2 M; protein concentration around 0.05%; 1 cm cell. The broken lines are extrapolations of the folded (N form) and the partly unfolded (F form) to constitute the rotations of the respective forms in the transition region in calculating the apparent equilibrium constant for the N-F transformation.

is related to the well examined N-F transformation (1). The folded and unfolded stages will be referred to as the N and the F stage, respectively. (b) The part which seems to be a linear function of pH, from pH 2.6 to pH 3.8, and which coincides with the electrostatic expansion will be referred to as the acid expansion according to convention (2, 7). From about pH 2.0 to 2.6, the conformation is independent of pH as measured by OR. This stage of the unfolding of HSA is called the acid-expanded stage and is here labeled E.

This finding is consistent with the picture of unfolding of HSA in the acid pH region as revealed by solvent perturbation (4) and free boundary electrophoresis (24). The "helix content"

calculated on basis of $(m')_{233}$ at the different stages and temperatures are listed in Table II. The E stage still contains 40% structure calculated as α helix. The values for the helix content of BSA for the same stages determined by Sogami and Foster (9) at ionic strength 0.1 M KCl are listed for comparison.

These authors report a large (9%) decrease in the helix content of BSA by acid expansion compared with the 1% obtained for HSA from the present results. The electrostatic interaction between the charged groups of a protein is strongly dependent on the ionic strength (25), but it is generally found that the interaction is already at a minimum at ionic strength 0.1 M. Thus, although additional experiments with HSA at conditions of lower ionic strength will be necessary, it appears probable that the observed quantitative differences between HSA and BSA are due to differences in conformational behavior rather than to the difference in the ionic strength of the experiments.

From inspection of Fig. 3 it is apparent that there is no stable intermediate between the N-F transformation and the acid expansion. Indeed the latter appears to be linearly dependent on pH. Again, this behavior is different from the behavior of BSA in 0.1 M KCl, where the N-F transition and the acid expansion transition are two distinctly separate reactions including a stable intermediate at pH 3.6 to 3.8 (2, 9).

By means of a linear extrapolation of the acid expansion to represent the values of $(m')_{233}$ for the F form and a horizontal extrapolation of the N form to represent the native state in the N-F transition region (Fig. 3), the equilibrium constant for the N-F transition has been calculated using the equations mentioned under "Methods."

To allow for the linear extrapolation of the F stage to the N-F transition region it is necessary to assume that this transition and the acid expansion are independent although simultaneous processes.

This assumption appears reasonable since it has been demonstrated that the COOH groups in the F stage, in contrast to the N stage, have normal titration properties and three COOH groups are already titrated at the isoionic pH of 5.4 (26). (This finding is actually based on experiments with BSA; however, it has previously been shown that the titration curves for HSA and BSA are similar (7).) Furthermore, due to electrostatic interactions, the wide distribution of the apparent pK values for the side chain COOH groups render the titration and the resultant acid expansion of the F stage a noncooperative process involving the entire molecule. As mentioned the N-F transition has by several techniques been shown to be blurred by the microheterogeneity of the albumin (9) which means that the equilibrium constant calculated will be apparent.

The midpoints of the N-F transition calculated from the K_{appNF} are displaced toward neutrality by increasing temperature (Table I). The apparent preferential binding of hydrogen ions, $\Delta\nu_{H^+appNF}$, is calculated to less than 3 protons per molecule of protein and is independent of temperature (Table I).

The temperature independence of $\Delta\nu_{H^+appNF}$ makes it probable that the N-F transition and the thermal unfoldings of HSA are independent processes.

A van't Hoff presentation of K_{appNF} (Fig. 4) shows that the curves at different pH have the same shape, which indicates that the apparent enthalpy changes ΔH_{app} in the N-F transformation is pH-independent. Furthermore, there is a temperature of maximum stability for the N stage at approximately 18° (Fig. 4).

The independence of pH for the variation of K_{appNF} with temperature (Fig. 4) indicates that the stability of the total

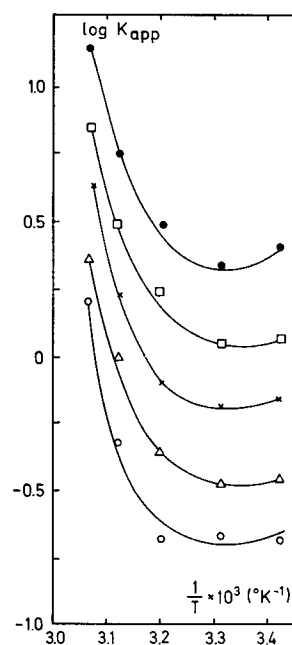


FIG. 4. Van't Hoff plots for the N-F transition of defatted HSA from the data of Fig. 3. ●—●, pH 4.1; □—□, pH 4.2; △—△, pH 4.4; ○—○, pH 4.5. The curve (×—×) is the average of the $\log K_{app}$ at the quoted pH and constitute the basis for the calculation of the enthalpies listed in Table I.

population of microheterogeneous species of HSA are equally influenced by temperature in the N-F transition. This means that in the N-F transformation the apparent equilibrium constant determined by OR must have the same variation with temperature as the true equilibrium constant. Therefore, the use of K_{appNF} in thermodynamic calculations should be valid when the calculation is dependent only on the temperature variation of the equilibrium constant. On this basis the ΔH_{app} values for the N-F transformation at different temperatures have been calculated from Fig. 4 and listed in Table I.

ΔH_{appNF}^0 is estimated to be 4 Cal per mole. Whether this value is the true standard enthalpy changes can only be decided if it is equal to the ΔH^0 determined calorimetrically (11). The calorimetrically determined ΔH^0 for the N-F transformation of BSA is 3.3 Cal per mole (27). When the accuracy of the methods is taken into consideration this value is of the same magnitude as ΔH_{appNF}^0 for HSA estimated in this work. However, as demonstrated it is not possible directly to compare the N-F transformation of BSA and HSA and the final conclusion regarding this point must await the calorimetric estimation of ΔH^0 for HSA.

The thermal unfolding of defatted HSA in 0.2 M KCl gives essentially the same picture for all the pH values examined (Figs. 2 and 5); the process is characterized by broad, continuous transitions with no detectable intermediate stages, and by temperatures of maximum contents of conformation below 0°. The unfoldings are not completed at 50°, at which temperature the reaction at pH values higher than 2.6 begins to be irreversible.

Some values for the helix contents, calculated at different temperatures and pH values, are listed in Table II.

The unfolding by guanidine HCl of HSA at pH 2.6, 5.6, and 9.2 is shown in Fig. 6. At pH 2.6, the unfolding starts from the E stage already at concentrations of guanidine HCl less than 1 M. The sigmoidal curve is nearly symmetrical about the value 4 M and approaches a minimum in the vicinity of 8 M guanidine HCl. At this concentration of guanidine HCl, the HSA is

completely unfolded and the random coil is restricted only by the S—S bridges (10).

The unfolding by guanidine HCl at pH 5.6 and pH 9.2 follows a similar pattern, but in these cases the unfolding is a multistage process including a cooperative transition over the range 1 to 2.5 M guanidine HCl. The cooperative part involves approximately twice the amount of structure participating in the N-F transition (Fig. 6). The completely disordered state is reached in 6 M guanidine HCl.

Thermal unfolding in different concentrations of guanidine HCl was performed at pH 7.5 (Fig. 7A) and pH 2.6 (Fig. 7B). When the temperature is changed from 5–50° there is at both pH values a build-up followed by a destruction of the HSA structure, and the temperature of maximum stability of the protein

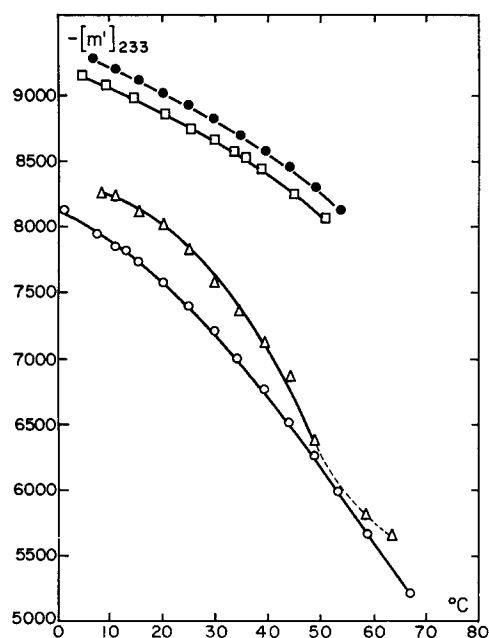


FIG. 5. The mean residue rotation at 233 nm of defatted HSA in variation of temperature at different pH. ●—●, pH 5.5; □—□, pH 7.5; △—△, pH 4.0; ○—○, pH 2.6. KCl, 0.2 M; protein concentration around 0.05%; 1 cm cell.

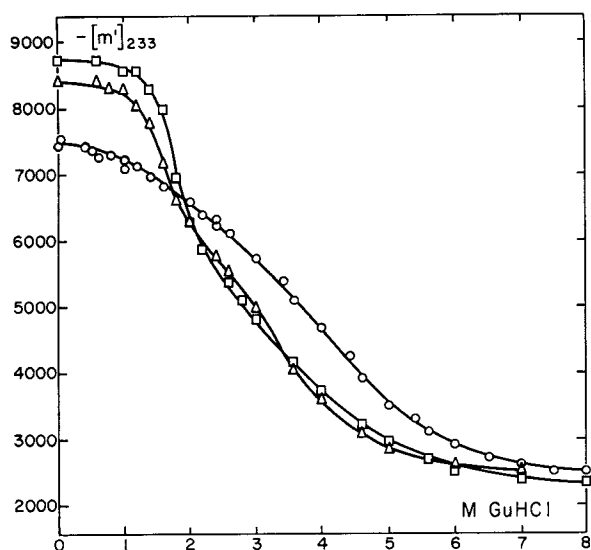


FIG. 6. The mean residue rotation at 233 nm of defatted HSA at different concentrations of guanidine HCl: at pH 9.2, △—△; at pH 5.5, □—□; at pH 2.6, ○—○. KCl, 0.2 M; protein concentration around 0.4%; 1 cm cell; temperature, 23°.

conformation is increasing with the guanidine HCl concentration. In 8 M guanidine HCl the mean residue rotation is continuously increasing with temperature in the range examined.

At pH 2.6 the unfolding of HSA by heat (Fig. 5) and by guanidine HCl (Fig. 6) are continuous processes. Furthermore the transition curve for guanidine HCl denaturation at this pH is symmetrical (Fig. 6). It is thus considered justifiable to extrapolate the variation of T_{max} with the molarity of guanidine HCl (Fig. 7B) to zero concentration. The best fitting curve to the experimentally determined T_{max} values of Fig. 7B has the equation $y = 7.9 \times 10^{0.1619} - 14.1$ and reveals a T_{max} for thermal denaturation of HSA at pH 2.6, 0.2 M KCl at -6° .

Probable explanations for the variation of T_{max} with guanidine HCl concentration are: (a) the denaturation of HSA is a multistage process with increase of the T_{max} of the intermediate stages as the unfolding proceeds. (b) The solution: protein-water-guanidine HCl constitute a complex mixture of interactions (protein-guanidine HCl, water-protein, water-guanidine HCl, water-water, etc.) which all are differently influenced by heat and concentrations. The variation of the optical rotation ac-

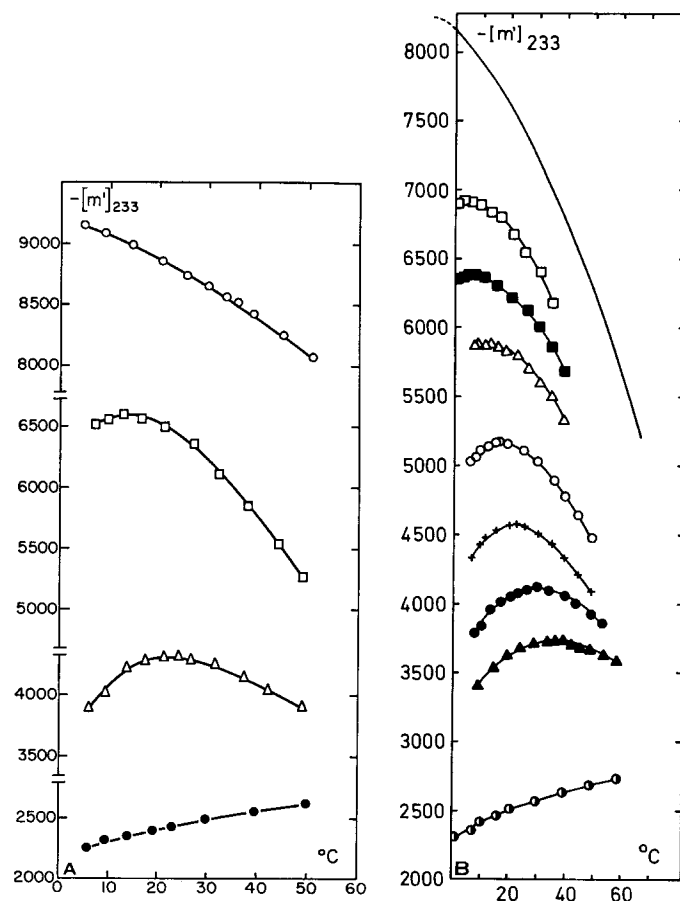


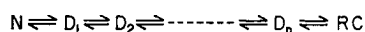
FIG. 7. The mean residue rotation at 233 nm of defatted HSA in variation of temperature at different concentrations of guanidine HCl; 0.2 M KCl; protein concentration around 0.4%; 1 mm cell. A, pH 7.5. Concentrations of guanidine HCl: ○—○, 0 M; □—□, 2 M; △—△, 3.5 M; ●—●, 8 M. B, pH 2.6. The unmarked line is in 0.2 M KCl, data from Fig. 5, concentrations of guanidine HCl: □—□, 2 M; ■—■, 2.5 M; △—△, 3 M; ○—○, 3.5 M; ×—×, 4 M; ●—●, 4.5 M; ▲—▲, 5 M; ●—●, 8 M. The measurement for each molarity of guanidine HCl and pH is made on the same sample, with control for reversibility after the experiment. The $(m')_{233}$ measured in 8 M guanidine HCl at various temperatures of this figure and Fig. 6 are used as the rotation of complete unfolded HSA in calculating the helix contents of Table II.

tually observed in Fig. 7 must be a competition between the temperature dependence of these interactions on the protein structure and the temperature dependence of the equilibrium constant for heat denaturation of HSA.

At present it is not possible to decide which of the explanations may apply to the continuous increase of T_{\max} in Fig. 7, and it is likely both might be acting at the same time.

Most work on denaturation of proteins has been made on low molecular weight proteins with known three-dimensional structure (10). One of the few larger proteins investigated is parmyocin (28); the transition profile by guanidine HCl at pH 7.3 shows a multistage process with more or less separated stable intermediates.

The reaction scheme connected with this type of denaturation processes is

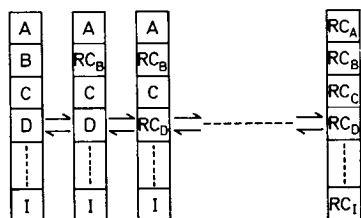


each step being the necessary basis for the subsequent unfolding. Neither in the thermal transition profiles of HSA nor in the unfolding by guanidine HCl at pH 2.6 can any stable intermediates be distinguished.

Attention will be drawn to another mechanism of unfolding of structural macromolecules proposed by Kallenbach (29) for the heat denaturation of low molecular weight RNA chains. According to this model the observed broad thermal transition profiles of low molecular RNA are the sum of the more or less independent unfoldings of different loops on the molecule with different enthalpies.

In analogy with this confirmed model the broad continuous transition profiles of HSA might be explained as the sum of simultaneous, more or less independent unfoldings of different sections of the protein with large differences in enthalpies of unfolding.

The reaction scheme according to this model is



where A, B, C, D, \dots, I represent different regions of the molecule. A support for this mechanism of reversible denaturation of HSA, besides the transition profiles for the thermal denaturation, is the demonstration that the thermal unfolding and the pH-dependent transitions are independent processes.

Hydrogen deuterium exchange measurements on defatted HSA show "parallel" displacements of the "relaxation spectra" by changes in temperature and pH (5). In the paper referred to it was stated that this finding could be explained either by a change in free energy of all measurable peptide groups or by local conformational changes of the protein shifting the exchange rate of a minor fraction of the peptide groups. The present demonstration of apparently low cooperativity and relative independence of the unfoldings do highly support the latter explanation and bring the hydrogen exchange results in accordance with the model of HSA proposed here.

The picture of the architecture of serum albumin proposed by Foster (1) and later given experimental evidence by several

authors (30–32) implies that the molecule contains three or four compact parts or "subunits" held together by the peptide backbone itself; the N-F transition being explained as the rupture of the weak bonds keeping the subunits in their steric position.

The present findings and the mechanism proposed for the reversible unfoldings of HSA are in good agreement with this model and the data furthermore strongly suggests that the subunits of the molecule and the structure which bring them into the native configuration relative to each other should have the ability to unfold simultaneously, with appreciable differences in enthalpies, and for some of the steps independent of each other.

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