11, Suppl. 24.
Sueoka, N. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 35.
Suyama, Y., and Bonner, W. D. (1966), *Plant Physiol. 41*, 383.
Suyama, Y., and Preer, J. R., Jr. (1965), *Genetics 52*, 1051.
Suyama, Y., Preer, J. R., Jr., and Bonner, W. D. (1965), *Plant Physiol. 40*, 95.
Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon Press.
Swift, H. (1965), *Am. Natural. 99*, 201.
Tewari, K., Jayaraman, J., and Mahler, H. (1965), *Biochem. Biophys. Res. Commun. 21*, 141.
Thomas, C. A., and Berns, K. I. (1961), *J. Mol. Biol. 3*, 277.

Titration of Salt-Extracted Human Skin Collagen*

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ABSTRACT: Hydrogen ion titrations were done on the undenatured and the heat-denatured (40°, 30 min, pH 6.5) forms of a purified and physically characterized salt-extracted collagen. Exposure to low pH of the undenatured soluble collagen resulted in the unmasking of 7.5 carboxyl groups/10^5 g. The release of these groups was not associated with detectable loss of helical structure. Either heat or high pH denaturation resulted in the unmasking of 5.0 carboxyl groups/10^5 g, 1.0 imidazole or α-amino groups/10^5 g, and 5.5 ϵ-amino groups/10^5 g.

The release of these groups was associated with loss of helical structure and separation of the protein to random coils. The unmasking of these ionizable groups is thought to result from the disruption of interchain bonds involving these groups. The precise chemical nature of these bonds cannot be determined from the titration data.

Titration studies have been done previously on collagen systems. Bowes and Kenton (1948) titrated intact collagen which had been extracted with 10% sodium chloride. Ames (1952) and Kenchington and Ward (1954) did extensive titrations of purified gelatin solutions. Jansson and Weaver (1964) have reported, in abstract form, titrations on collagen and gelatin extracted from rat tail tendon by the method of Dumitru and Garrett (1957), but these titrations were carried out only from pH 1 to 5. At the present time, however, no full pH range titrations have been reported for soluble collagen molecules in the undenatured and denatured states. The major objective of this work was to compare the numbers of groups titrated in the undenatured state with the numbers of groups titrated in the denatured state for collagen.

Salt-extracted molecules were chosen for this investigation because these molecules apparently represent an early phase in the maturation of collagen (Jackson and Bentley, 1960.) During the extraction procedure, intermolecular bonds were broken. Titration curves were obtained on the undenatured solubilized collagen molecules. During heat denaturation, the interchain bonds were broken. Titration curves were then obtained on the component polypeptide chains. Differences between the kinds and numbers of groups titrated in the two states gave the type and numbers of groups unmasked.

Experimental Procedure and Characterization of Soluble Collagen

Extraction Procedure. Body skin was obtained at autopsy within 8 hr of death from full-term newborns. In no specimen was gross or microscopic evidence of decomposition present. All preparatory procedures were carried out at 5° or less. The epidermis and subcutaneous fat were scraped off with a sharp blade; microscopic sections confirmed complete removal. The dermis was fragmented with a mechanical grinder and washed thoroughly and repetitively with large amounts of triple-distilled water for 48 hr, to remove readily soluble material. The washed dermis was extracted with 1.0 M NaCl (1:4, wet weight:volume) pH 7.4 for 48 hr, and the supernatant fluid was collected following centrifugation at 59,000*g* for 1 hr. The dissolved soluble collagen in the supernatant was precipitated by dialysis *vs.* at least 1000 times its volume of 5 M NaCl and collected by centrifugation at 59,000*g* for 1 hr. The precipitate was dissolved in 1 M NaCl and dialyzed *vs.* at

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least 1000 times its volume of 1 M NaCl for 24 hr. The resulting solution was centrifuged at 105,000*g* for 30 min, to remove insoluble material. The collagen was then reprecipitated by dialysis *vs.* 5 M NaCl. The precipitate was collected by centrifugation and redissolved with 1 M NaCl. Any residual precipitate was removed by centrifugation.

The preparations of salt-extracted collagen were next dialyzed *vs.* water to remove the sodium chloride. The salt-free collagen precipitated in the dialysis bag and was then dialyzed *vs.* 0.5 *m* KCl to introduce the new solvent and to resolubilize the collagen. The 0.5 *m* KCl solution was clear, colorless, and free of insoluble residue. Portions of the salt-soluble collagen were also dialyzed *vs.* citrate buffer for certain physical measurements which are detailed below.

Amino Acid Analyses. Complete amino acid analyses were done according to the method of Spackman *et al.* (1958) to determine the distribution of the amino acids and to judge the purity of the preparations. The complete absence of cystine and cysteine and the presence of tyrosine in the amount of two residues/10^5 g were indicative of a preparation free from contaminating proteins in detectable amounts. The complete amino acid analyses are not important for the interpretation of the results pf the titration data, and for that reason will not be presented here. The distribution of amino acids "characteristic" of collagen and of those residues which contain titratable reactive groups on side chains was as follows (results in residues/10^5 g, mean residue weight 91.5 g): hydroxyproline, 101; proline, 137; glycine, 366; glutamic acid, 80.9; aspartic acid, 48.2; combined side-chain carboxyls, 129; amide nitrogen, 39; histidine, 5.5; lysine, 29.7; hydroxylysine, 6.5; combined side-chain amino groups, 36; arginine, 54.6.

Physical Characteristics. The physical characteristics of the salt-soluble collagen were determined under conditions similar to those in which the titrations were performed, *i.e.*, both in undenatured and denatured states and in two different solvents. Analytical ultracentrifugal, viscosity, and optical rotation measurements were carried out on a known quantity of the purified salt-extracted collagen, dialyzed *vs.* 0.15 M citrate buffer, pH 3.7. Optical rotation measurements and the titration of the salt-extracted collagen were measured in 0.5 *m* KCl. Denaturation of the soluble protein by heating to 40° for 30 min was done both in citrate buffer and in 0.5 *m* KCl at pH 6.5. The protein did not form a solid gel after denaturation under the above conditions. The low protein concentration, 3 mg/ml, may be the reason for this fact.

ULTRACENTRIFUGATION. The analytical ultracentrifuge studies were done in a Spinco Model E ultracentrifuge equipped with a phase plate and temperature control. A speed of 50,740 rpm and temperatures of 20° for the undenatured collagen and 35° for the denatured collagen were used. A longer cell path length of 30 mm was employed. With this longer path length, lower concentrations could be employed and more accurate sedimentation coefficients were obtainable following extrapolation to infinite dilution. Furthermore, following denaturation, the areas under the ultracentrifuge patterns of the α and β components were measurable at concentrations lower than are usually achieved; this was important to minimize the Johnston–Ogston effect (1946). The areas under the curves were determined by magnifying the ultracentrifuge patterns 25 times and measuring the areas with a planimeter. The $\bar{V}$ was determined by both direct measurement and by calculation from the amino acid analyses. Values of 0.706 cc/g (direct measurement) and 0.704 cc/g (amino acid analyses) were obtained. A $\bar{V}$ value of 0.705 cc/g was used in calculations.

OPTICAL ROTATION was measured with a Rudolph photoelectric polarimeter, Model 200, equipped with an oscillating polarizing prism at the sodium D line in a 10-cm path length water-jacketed tube maintained at 20 ± 0.1°. The instrument was adjusted accurately with a quartz control plate obtained from the U. S. National Bureau of Standards. The optical rotation was measured both in citrate buffer and in the same solvent (0.5 *m* KCl) as that used in the titration.

VISCOSITY was measured in a calibrated Cannon–Manning semimicrocapillary viscometer in an insulated water bath having an electronic temperature control, monitored by a Beckmann thermometer to ±0.003°. The kinetic energy correction, determined by measuring the flow of water at two different temperatures, was insignificant. The volume of sample used was 0.45 ml and the flow time for water was 247 sec at 20°.

CONCENTRATIONS were determined by Conway (1957) diffusion techniques preceded by Kjeldahl digestion. To calculate the dry weight of collagen, a value of 18.7% nitrogen, obtained from amino acid data, was used. Reproducibility of the results by the Conway procedure was ±2%. In critical experiments where slight errors in concentration could lead to substantial errors in experimental results (as in the determination of the weight of the material titrated), protein concentrations were obtained directly from the dry weight of the sample. The special precautions used in these procedures are given under "Titration Procedure."

SUMMARY. The physical characteristics of the 1 M salt-extracted collagen used in the titration were as follows: undenatured collagen, sedimentation coefficient: $s^0_{20,w}$ = 3.15 S; specific rotation: $[\alpha]^{20}_D$ 415 ± 30°; limiting viscosity number (g/ml) = 1360 ± 30; denatured collagen, specific rotation: $[\alpha]^{20}_D$ 110 ± 10°; limiting viscosity number (g/ml) = 40 ± 5. The ultracentrifuge patterns, corrected for Johnston–Ogston (1946) effect, of the denatured collagen showed 90% α component and 10% β component by weight. No other peaks were detectable.

Titration Procedure. Potentiometric measurements were made with a Radiometer TT1 titrator, a titragraph SBR 2/SBU 1, Radiometer G-202B glass electrodes, and K130 reference electrodes. These electrodes were restandardized after each run to ensure that no drift had occurred during titration. The reagents used were as follows: 0.1 M CO_2-free KOH standardized with potassium acid phthalate to 0.33 part per thousand by six determinations, and then used to stand-

ardize 0.1 M HCl to 0.21 ppt by six determinations. KCl was used to obtain an ionic strength of 0.5. Temperature was controlled to ±0.1° with a water-jacketed titration flask. Room temperature was 20 ± 2°. Air was excluded from the reaction vessel with a stream of nitrogen moisturized at the temperature of titration. Stirring was rapid and continuous.

Standardizing Procedure. Of particular importance was standardizing the experimental technique. Titrations done on 0.5 *m* KCl solutions at 20 and 5° were sufficiently perfected that the titrations were predictable within a tolerance of ±0.01 pH unit at pH or pOH values <3.5. Reproducibility of this degree was necessary to quantitatively detect the hydrogen ion bound by the relatively small amounts of collagen that can be dissolved. Titration curves traced by the titragraph, under identical conditions, were virtually superimposable.

Experimental values for $-\log_{\gamma'H^+}$ and $-\log_{\gamma'OH^-}$ (at 20 and 5°) were calculated, as described by Tanford (1955), from the information obtained from control titrations with protein absent. These activity coefficients were used in the calculations of the final protein titration curves.

Experimental Procedures. The quantity of collagen titrated was determined as follows: portions of the stock solution to be titrated were measured with a syringe-type microburet in order to avoid pipetting errors due to the viscosity of solutions. After titration was completed, the samples were quantitatively transferred to dialysis bags, dialyzed *vs.* at least 1000 times its volume of water, and transferred to a weighing container (the dry weight of which had already been determined). The water was first removed from the sample by evaporation, the sample dried in a vacuum oven at 70° for 24 hr and then weighed on an analytical balance (sensitivity ±0.02 mg). The drying process was repeated until a constant weight was obtained (±0.05 mg). The analytical balance and vacuum oven were housed together in a large air-tight container which was filled and circulated with dry nitrogen. Samples were transferred from the oven to the balance without exposure to atmospheric humidity. After weighing, the samples were ignited in a muffle furnace and their ash weight was determined. The collagen concentrations of separate samples of the original solution determined in this way deviated from each other by <1%.

Titrations on soluble collagen were carried out in the following manner: (1) Fresh aliquots of undenatured collagen solution were used for measurements on acidic and basic sides and for different temperatures. (2) Each sample was titrated from a pH near seven to the respective extreme of pH. (3) The sample was then back titrated to the starting pH. (4) The sample was then retitrated for comparison. (5) Samples were heat denatured, and procedures 1–4 were repeated. Each titration from neutral to an extreme pH was extended over a 3-hr period. (6) Steps 1–5 were then repeated at 5°, on separate samples. Optical rotation was measured after titrations, to monitor the configurational status of the molecules.

Calculations. Experimental titration curves were first constructed with pH 6.5 as the arbitrary point of zero hydrogen ion binding. Values of H^+ bound/10^5 g and OH^- bound/10^5 g were calculated from (a) the continuous protein titration curves produced by the titragraph of volume of titrant added *vs.* pH, (b) the molality of the standard titrant solutions, (c) the experimental values of $-\log_{\gamma'H^+}$ and $-\log_{\gamma'OH^-}$, at 20 and 5°, (d) the values of K_w, at 20 and 5° and in 0.5 M KCl, obtained by Harned and Owen (1958), (e) the density of the solutions at the temperature under consideration, and (f) the dry weight of the collagen titrated. The methods and assumptions outlined in detail by Tanford (1955, 1962) were utilized. The most important assumption made in these calculations was that at high ionic strength (experimental value, $\mu = 0.50$) the activity coefficients of hydrogen or hydroxide ions was not affected by the presence of protein in the system.

From the curve constructed with points calculated in the above manner, the acid end point or the point of maximum hydrogen ion binding was determined. For each titration, the point of zero hydrogen ion bound was moved from its arbitrary position at pH 6.5 to the acid end point, and the units of the ordinate were changed to moles of H^+ dissociated/10^5 g. The reassignment of the zero point does not change the relative values of the individual points; it simply facilitated comparison between different titration curves.

Discussion of Errors. The *absolute* error predicted for individual titration curves was calculated from the predicted errors in the individual experimental steps. It was found to range from ±2–3 groups/10^5 g at the pH extremes to less than one group/10^5 g at near-neutral pH values. The major aim of this investigation, however, was to quantitate *differences* between the numbers of titratable groups. This is done by subtracting the values of two comparable points on two different titration curves over the entire pH range of the titrations. The absolute error produced will always be in the *same direction*, at comparable points, on separate titrations. When the difference between the values at two points on different titration curves is computed, the resultant error in these differences is lower than the absolute error at these two points. This is due to the fact that constant errors (concentration of protein, normality of standard solutions, etc.), which contribute substantially to the absolute error of any individual titration curve, tend to be canceled.

Considerations in Analyzing the Titration Curves. The first problem in analysis of any titration curve is the allotment of specific areas of hydrogen ion binding to specific reactive groups. In collagen, this task is immensely simplified by the presence of only three types of titratable reactive groups in amounts large enough to accurately quantitate. These three types are side-chain carboxyls, imidazoles of histidine, and ε-amino groups of lysine and hydroxylysine.

The influence of other types of reactive groups on the titration curves was minimal for the following reasons. Amino acid analysis of our preparation showed no cysteine. There were only two residues of tyrosine/10^5 g.

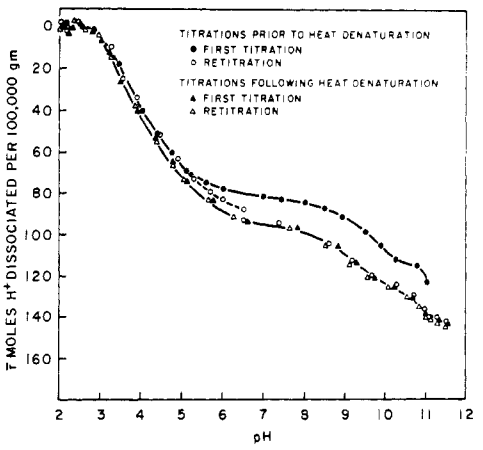


FIGURE 1: Titration curves for 1.0 M NaCl-extracted human skin collagen at 20° and $\mu = 0.50$.

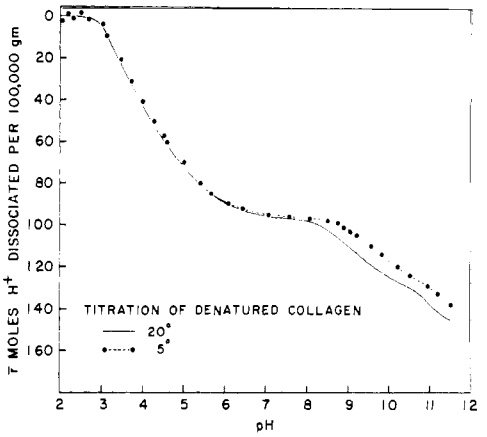


FIGURE 2: Effect of temperature on the titration curves of heat-denatured salt-extracted collagen at $\mu = 0.50$. Experimental points for 20° titration are given in Figure 1. Experimental points shown for 5° titration represent the average of the titration and the retitration.

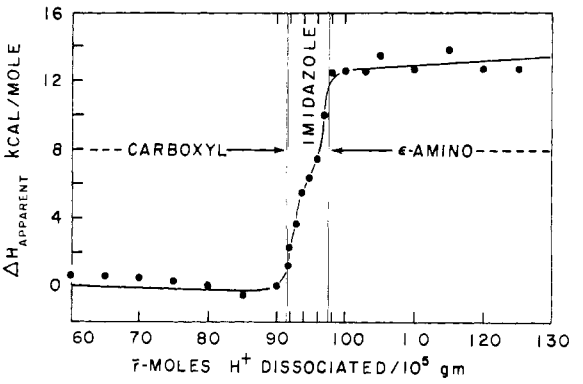


FIGURE 3: ΔH_{app} *vs.* $\bar{r}$ moles of H^+ dissociated/10^5 g of collagen. Points were calculated from the titration curves (Figure 2) of salt-extracted collagen after heat denaturation.

These phenolic groups will titrate in the basic region of the curve with the ε-amino groups of lysine and hydroxylysine and contribute about a 5% positive error if not taken into account. By subtracting the two phenolic groups from the number of groups titrated in the ε-amino pH range, the number of ε-amino groups will be obtained. Further simplification of analysis is made possible by the paucity of α-amino and carboxyl end groups in collagen, there being no more than one group/10^5 g of each of these groups and possibly even fewer (Stevens and Tristram, 1962). Lastly, guanidyl groups of arginine are usually found to titrate at pH values outside the range of our titration.

To facilitate the analysis, it was assumed that the solubilized salt-extracted collagen molecule is completely penetratable by aqueous hydrogen ion. Collagen molecules are thought to be rigid rods with a diameter of only about 15 A, and for this type of molecule this assertion could not be grossly in error.

Results

The titration curve of the denatured form of the molecule was reversible and reproducible, whereas the undenatured molecule underwent irreversible alteration at extremes of pH (Figure 1). For this reason, the denatured curves are considered first.

Denatured Collagen

Group Assignment. The allotment of areas on the titration curves of denatured collagen to various reactive groups was made from changes in the heats of ionization. Apparent changes in heat of ionization for titrated groups were calculated from the 20 to 5° titration curves (Figure 2). The following formula was utilized (Steinhardt and Beychok, 1964)

$$\Delta H_{app} = 2.303R \frac{T_1 T_2}{(T_1 - T_2)} (pH_2 - pH_1)_{\bar{r}}$$

where $\bar{r}$ indicates that pH values were measured at the same state of titration (*i.e.*, the same value of $\bar{r}$, see Figure 2) at each temperature.

When ΔH_{app} was plotted *vs.* $\bar{r}$, the curve seen in Figure 3 was produced. Two sharp breaks are seen to occur at $\bar{r} = 90$–93 and $\bar{r} = 97$–99. The usual ranges for ΔH_{app} are as follows: ±1.5 kcal/mole for carboxyl; 6.5–7.5 kcal/mole for imidazole; and 11–13 kcal/mole for ε-amino groups (Cohn and Edsall, 1943). It is therefore clear that the first break represents the transition from carboxyl to imidazole and the second from imidazole to ε-amino groups. Although the imidazole groups were not present in high enough quantity to cause a plateau in the range of $\Delta H_{app} = 6.5$–7.5 kcal/mole, a deviation in the curve is seen in this area. The end-point lines (solid lines in Figure 3) were placed at what appeared to be the most appropriate cutoff point

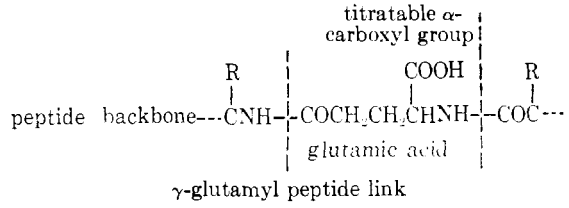
TABLE I: Titration Data for Denatured Salt-Extracted Collagen.[a]

Group Type	pH Range of Titration	No. of Groups Titrated/ 10^5 g	Amino Acid Anal	pK_{obsd}	ΔH_{app} (kcal/mole)
Side-chain carboxyls	2–6.25[b]	91.5	90.0 (129–139 amide N)	4.1	±1.0
Imidazole	6.25[b]–8.00[b]	6.0	5.5	6.9	+6.5
ϵ-Amino and phenolic	8.00[b]–11.5[c]	45.5[d]	38	9.8	+12.5 to +13.75
Guanidyl	Could not be evaluated				

[a] Titration at 20 ± 0.1°. [b] End points derived experimentally from ΔH_{app} curve. [c] Cannan (1942) criteria. [d] See text for explanation of discrepancy of titrated groups and analysis.

(*i.e.*, $\bar{r}$ = 91.5 and 97.5). The breaks in the ΔH_{app} curve are sufficiently sharp so that the end-point lines (solid lines) cannot be moved by more than ±1 group without producing an obvious misinterpretation of the data.

The ΔH_{app} curve allows division of the curve for denatured protein (Figure 1) into three areas at $\bar{r}$ values corresponding to the end-point lines in Figure 3. The first area, representing groups $\bar{r}$ = 0–91.5 (total 91.5 groups) and a pH range from 2 to 6.25, contains titrated carboxyl groups.[1] The second area, $\bar{r}$ = 91.5–



97.5 groups (total six groups), pH range 6.25–8.00, represents imidazole and α-amino titration. Finally, the third region represents titrated groups from pH 8 to 11.5, $\bar{r}$ = 97.5–143 (total 45.5 groups). This third region represents the titration of the two phenol groups known to be present by amino acid analyses and the ϵ-amino groups. The results are tabulated in Table I.

Comparison of the Results of Group Assignment with Amino Acid Analyses. ACIDIC GROUPS. Amino acid analyses showed the presence of 129 residues of aspartic and glutamic acids/10^5 g of collagen. If no carboxyls were masked, one would predict the titration of 129 groups in the acid range. Titration after heat denaturation yielded 91.5 groups. This left 37.5 carboxyls still masked (129 − 91.5). This was compatible with the 39 amide nitrogen/10^5 g which would mask this number of carboxyls in glutaminyl and asparaginyl residues. Thus, all side-chain carboxyl groups known to be present were accounted for.

NEUTRAL GROUPS. Amino acid analysis showed the presence of 5.5 histidine residues/10^5 g. Six groups were titrated in this range, thus accounting for the imidazole groups within an error of 0.5 group/10^5 g.

BASIC GROUPS. Alkaline end point of the basic range could not be determined from ΔH measurements. A pH of 11.5 was suggested as the alkaline end point for ϵ-amino titration by Cannan (1942), and was used in determining the quantity of basic groups shown in Table I. Kenchington and Ward (1954) and Ames (1952) also used pH 11.5 as the alkaline end point for their titrations of acid-processed gelatin. These investigators found 43 basic groups titratable to this end point (our results, 45.5 groups). From amino acid analyses, one would only predict 36 ϵ-amino and two phenolic groups or a total of 38 groups for this region. Since two other investigators, besides ourselves, have titrated more than the expected number of groups in this region, it is our feeling that these extra titratable groups are due to the titration of additional groups of another type, rather than to error. It is possible that several of the guanidyl groups of arginine titrate on the "tail end" of the usual ϵ-amino range because of electrostatic or inductive factors. This would suggest that the actual end point of ϵ-amino titration has a pH somewhat <11.5; regardless of the interpretation, the ϵ-amino groups are completely titrated. The uncertainty of the precise ϵ-amino end point did not affect the computation of the numbers of unmasked groups, because the undenatured protein started to undergo denaturation at pH 10.75. Beyond this pH, additional groups could not be determined.

IN SUMMARY. The quantitative agreement between titration and amino acid analysis data indicates that no bonds (except the amides of aspartamide and glutamide)

[1] The carboxyls titrated in the denatured state will be expected to contain a mixture of β- and γ-side-chain carboxyls and the α-carboxyls which are available for titration as a result of γ-glutamyl peptide links as described by Franzblau and co-workers (1963). Even though such α-carboxyls are not side-chain carboxyls in the usual sense of the term, functionally they will be exposed at the "side" and thus must be considered as possible participants in cross-links. Therefore, the term "side-chain carboxyl" will be applied to all these groups, with the reservation in mind that some of these are actually α-carboxyls exposed as a result of γ-glutamyl peptide linkages in the polypeptide backbone.

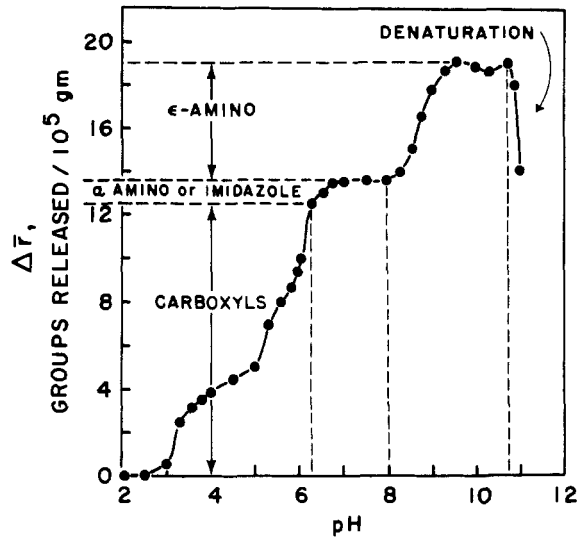


FIGURE 4: Groups released in moles/10^5 g of collagen.

remain between reactive groups on side chains after denaturation. This result agrees with the independent observation that salt-extracted collagen denatures into predominantly α-type chains which are thought to be single and noncross-linked.

The pK Observed. The pK observed (the point at which one-half of the groups in each range are titrated) is nothing more than a rough indication of the weighted mean pK of the groups titrated in that region. Keeping in mind the approximate nature of this quantity, a few observations were made.

For the carboxyl region of the denatured curve, pK_{obsd} = 4.1. If all the groups were either β- or γ-carboxyls, the expected pK would be 4.6. The low value of the pK_{obsd} (4.1) may be due to the presence of α-carboxyls (pK_{int} = 3.3) (Steinhardt and Beychok, 1964) of the glutamic acid residues which participate in the γ-glutamyl peptide links. It would, however, also be possible to account for this anomaly in the pK_{obsd} on the basis of electrostatic interactions. Values close to the one observed have been found in other proteins. (keratin pK_{int} = 4.2 in high salt; ovalbumin pK_{int} = 4.3; serum albumin pK_{int} = 4.0; trypsin pK_{int} = *ca.* 3.5; Steinhardt and Beychok, 1964).

For the imidazole groups, pK_{obsd} = 6.95. This is close to the value obtained in other proteins (chymotrypsinogen pK_{int} = 6.7; ovalbumin pK_{int} = 6.7; ribonuclease pK_{int} = 6.5; serum albumin pK_{int} = 6.9; Steinhardt and Beychok, 1964).

For the basic region the pK_{obsd} depends on the end point of ϵ-amino titration. If the alkaline end point is assumed to be pH 11.5, then pK_{obsd} = 9.8. If, as was pointed out, the actual end point is somewhat lower than pH 11.5, the pK_{obsd} would also be lower. In model polypeptides (Steinhardt and Beychok, 1964) for the ϵ-amino group the pK_{int} = 10.2; the low value of the pK_{obsd} can only partially be explained by the presence of two phenolic groups, pK_{int} = 9.6, and 6.5 ϵ-amino groups of hydroxylysine, pK_{int} = 9.6. It must therefore be assumed that the electrostatic or inductive forces are acting, which tend to lower the pK_{obsd}. The value of the pK_{obsd} (9.8) is not, however, so low as to be unprecedented in other proteins (conalbumin pK_{int} = 9.64; zinc free insulin pK_{int} = 9.6; β-lactoglobulin pK_{int} = 9.9; paramyosin pK_{int} = 9.65; serum albumin pK_{int} = 9.8; Steinhardt and Beychok, 1964).

Undenatured Collagen

Changes in heats of ionization could not be used in analyzing the titration curves of the undenatured material because irreversible changes occurred during the titration process which render thermodynamic approaches tenuous. These undenatured collagen titration curves shown in Figure 1 could have been divided into various titratable groups by using other criteria, but precise assignment is unnecessary. As will be seen subsequently, curves based on differences between the various titration curves exhibit clear end points which make possible the accurate identification of the unmasked groups.

The retitration prior to heat denaturation (Figure 1) was not only different from the initial titration but was also dependent upon respective exposures either to acid or to base in the preceding titration. In the case of previous exposure to high pH, the retitration was identical with the titrations of heat-denatured material. On the other hand, in the case of previous exposure to low pH, the titration curve fell between the initial and the reversible titration of the heat-denatured protein (Figure 1). The significance of these findings will become clear in the discussion.

Discussion

Analysis of Groups. The major objective of this work was to compare the numbers of groups titrated in the undenatured state with the numbers of groups titrated in the denatured state. Titrations were done in high salt concentrations, 0.5 M KCl, where electrostatic interactions are minimized and where the contribution of the protein to ionic strength is negligible. The use of relatively low protein concentration, 3 mg/ml, reduced protein–protein interaction. It would be of interest to examine the titration of the soluble collagens at varying concentrations to study the possible effects of aggregation. However, the range of concentrations at which soluble collagen titrations are feasible is narrow because of the low solubility at higher concentrations and because of increasing titration errors with lower concentrations. Conclusions concerning the bonds themselves must be made indirectly from the groups released and from the conditions (pH and temperature) which brought about their release.

All the groups released are represented graphically in Figure 4. This figure was produced by plotting the $\bar{r}$ differences between the initial titration prior to heating and the reversible curve of the denatured protein *vs.* pH. Figure 4 has three distinct regions. The first region (pH 2–6.25) demonstrates the presence of 12.5 car-

boxyls. The second region (pH 6.25–8.00), which represents any imidazole or α-amino groups released, shows only one unmasked group. The third region (pH 8.00–10.75) shows the presence of 5.5 ϵ-amino groups unmasked.

The presence of a plateau region between the acid and basic regions, in Figure 4, permits the very accurate identification of the groups. The total number of groups assigned to carboxyl or ϵ-amino groups, respectively, will not be changed by more than one group, as long as the end points of these groups are placed between pH 6.25 and 8.00.

ACIDIC GROUPS UNMASKED BY EXPOSURE TO LOW pH. The original titration on the undenatured material (Figure 1) was done from pH 6.5 to 2. On retitrating the same sample from pH 6.5 to 2, additional carboxyl groups were available for titration. This indicated the presence of "low pH" labile bonds. The groups released are shown graphically in Figure 5 and were obtained by plotting the differences between groups titrated during the first titration and groups titrated during the retitration. As can be seen from Figure 4, the groups released by exposure to low pH account for only 7.5 of the total of 12.5 carboxyl groups shown in Figure 4. Since these bonds are acid labile, some might have been broken during the initial titration to pH 2. Therefore, it is possible that more than 7.5 carboxyls were initially involved in bonding prior to *any* titration. These carboxyls released during the initial titration would not appear on the $\Delta\bar{r}$ curve because they would be indistinguishable from the unbonded carboxyls.

The optical rotation of the protein was measured after exposure to low pH, but *before heating*. It was found to be identical with that measured before titration ($[\alpha]_D^{20}$ $-410°$). Thus, regardless of the exact number of these acid-labile bonds, their disruption does not result in a detectable loss of helical configuration.

ACIDIC GROUPS UNMASKED BY EXPOSURE TO HEAT. The remaining 5.0 carboxyl groups of the total 12.5 were released only after the protein was heated to 40° for 30 min (Figure 4). Measurements of optical rotation confirmed loss of helical structure ($[\alpha]_D^{20}$ $-110°$) at this point. These groups released, shown in Figure 5, are represented by the $\Delta\bar{r}$ for the retitration prior to heating and the titrations after heating. A retitration of the heat-denatured material showed no additional release of groups.

SUMMARY OF ACIDIC GROUPS RELEASED. In the undenatured state, there are, firstly, low pH or acid-labile bonds which released at least 7.5 carboxyl groups, and secondly, heat-labile bonds which released 5.0 carboxyl groups. Furthermore, the acid-labile bonds might be broken without interfering with the helical structure, but the rupture of the heat-labile bonds was associated with loss of helical configuration and separation of the protein into noncross-linked α chains.

NEUTRAL GROUPS RELEASED. Only one group was released from pH 6.25 to 8.00 (Figure 3). We cannot distinguish whether this was an imidazole or an α-amino group.

BASIC GROUPS RELEASED. Lastly, the basic side of the titration was considered. Figure 1 shows the first titration progressing in a regular fashion until pH 10.75 whereupon there is rapid titration of groups. The optical rotation of the protein was measured and was found to be $[\alpha]_D^{20}$ $-110°$, thus indicating that the protein underwent denaturation into α chains simply by exposure to high pH. The retitration that was done on the protein, previously exposed to high pH, was found to be identical with the titration curves of the protein which had been heat denatured (Figure 1). Heat denaturation, therefore, did not unmask any additional groups. This result was not unexpected, since the optical rotation was the same ($[\alpha]_D^{20}$ $-110°$) whether the protein was denatured by heat or denatured by high pH, thus indicating that the protein was in the form of α chains in both cases. In addition, optical rotation measured at the completion of the experiment showed no elevation, indicating that no measurable renaturation had occurred during experimentation.

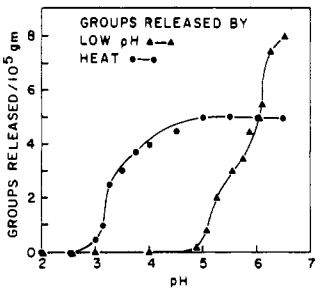


FIGURE 5: Carboxyl groups divided into those unmasked after exposure of low pH (*i.e.*, involved in acid-labile bonds) and those unmasked only after exposure to heat (*i.e.*, involved in heat-labile bonds).

Figure 4 indicates graphically what occurred in terms of groups released. From pH 8.00 to 10.75, 5.5 ϵ-amino groups are masked in the undenatured molecule. Between pH 10.75 and 11.0 all of these masked groups become available for titration. The optical rotation ($[\alpha]_D^{20}$ $-110°$) indicated that the protein was denatured; analytical ultracentrifugation showed that the collagen had been broken into single α chains.

These 5.5 basic titratable groups were not derived from hydrolysis of α-peptide linkages in the protein backbone, for the following reasons: firstly, neither the denaturation conditions, 40°, 30 min, pH 6.5, nor the high pH (pH 11.5) at 20° for <30 min were strong enough to hydrolyze these bonds. Secondly, if the α chains had been randomly hydrolyzed to yield 5.5 α-amino groups/10^5 g of collagen, one would expect the appearance of smaller components in the analytical ultracentrifugation patterns. This did not occur.

It was unlikely that alkaline hydrolysis of glutaminyl and asparaginyl residues contributed significantly to the release of these basic groups, for the following reason: Gallop *et al.* (1959) and de la Burde *et al.* (1963) found minimal hydrolysis of these bonds, while using conditions (1 M hydroxylamine, 40°, and high pH) far more

TABLE II: Groups Released.

Group Type	Groups/10^5 g	Condn Releasing Groups	Protein Configuration
Carboxyl	12.5 total		
	7.5	Low pH (~2)	Helical
	5.0	Heat (40°, 30 min, pH 6.5)	α chains in random coils
Imidazole and α-amino	1	Heat (40°, 30 min, pH 6.5)	α chains in random coils
ϵ-Amino	5.5	Heat (40°, 30 min, pH 6.5) or high pH (~11.0)	α chains in random coils
Guanidyl	Could not be evaluated		

likely to hydrolyze the bonds than our conditions (pH 11.5 and 20°). Furthermore, their protein was exposed to these conditions over a much greater time period than that of the titrations.

Further support for the identification of these unmasked groups as ϵ-amino groups can be derived from the fact that other investigators have produced evidence for the masking of the ϵ-amino groups in collagen. For example, Betheil and Gallop (1960) found that 13% of the ϵ-amino groups of lysine and 25% of the ϵ-amino groups of hydroxylysine (total 4.3 ϵ-amino groups/1000 residues) were not guanidinated in ichthyocol (temperature 5°, pH 9.3). If their results are converted to groups/10^5 g the value of 4.7 masked ϵ-amino groups is obtained. This is strikingly similar to our value of 5.5 ϵ-amino groups released. Franzblau (1962) found 12% of the ϵ-amino groups not available to FDNB[2] in a collagenase digestion of ichthyocol. The data on groups released are summarized in Table II.

Additional Considerations of Titration Data. THE ACID-LABILE TYPE OF BOND. The release of 7.5 carboxyls after exposure to low pH indicates the presence of at least 7.5 acid-labile bonds. No comparable groups are released on the basic side. It cannot be concluded from the titration data what type of bond was broken. In view of the fact that groups were released without loss of helical structure, it seemed unlikely that they were interchain cross-links.

THE HEAT- AND HIGH pH-LABILE TYPE OF BOND. In contrast to the acid-labile bond, the groups released after exposure to heat or high pH are associated with loss of helical structure and separation of the collagen molecule into its component chains. These groups therefore have much greater potentiality as possible participants in interchain cross-links. The fact that the carboxyl and ϵ-amino groups are released in equimolar quantities (5.0 carboxyl *vs.* 5.5 ϵ-amino) may mean that both bonding sites were derived from the rupture of a single bond.

It is unlikely, however, that covalent bonds involving a side-chain carboxyl and an ϵ-amino group of lysine would be broken by warming to 40° at neutral pH for 30 min. Furthermore, the denaturation of collagen and release of α chains from salt-soluble collagen can also be accomplished by cold 5 M guanidine, which argues against the presence of covalent interchain bonds in collagen lacking β components. Considering the conditions used for denaturation, it can only be concluded that the bonding sites are masked by relatively weak bonds, such as electrostatic bonds or very labile covalent bonds.

Masking could also occur in hydrophobic regions, which might not be penetrable to hydrogen ion. Although such regions are not thought to be present in monomeric solutions, the formation of aggregates could result in this type of masking, and cannot be excluded by experimental data. The information presented here concerning the quantity and identity of the titratable groups released during denaturation may, however, become valuable to other investigators as more data become available.

[2] Abbreviation used: FDNB, fluorodinitrobenzene.

References

Ames, W. M. (1952), *J. Sci. Food Agr. 3*, 579.

Betheil, J. J., and Gallop, P. (1960), *Biochim. Biophys. Acta. 45*, 598.

Bowes, J. H., and Kenton, R. H. (1948), *Biochem. J. 43*, 358.

Cannan, R. K. (1942), *Chem. Rev. 30*, 395.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions, New York, N. Y., Reinhold, Chapter 20.

Conway, E. (1957), Microdiffusion Analyses and Volumetric Error, 4th ed. C. Lockwood, London.

de la Burde, R., Peckham, L., and Veis, A. (1963), *J. Biol. Chem. 238*, 189.

Dumitru, E. T., and Garrett, R. R. (1957), *Arch. Biochem. Biophys. 66*, 245.

Franzblau, C. (1962), Ph.D. Dissertation, Albert Einstein College of Medicine, Yeshiva University, New York.

Franzblau, C., Gallop, P., and Seifter, S. (1963), *Biopolymers 1*, 79.

Gallop, P. M., Seifter, S., and Meilman, E. (1959), *Nature 183*, 1659.

Harned, H. S., and Owen, B. B. (1958), Physical Chemistry of Electrolytic Solutions, 3rd ed, New York, N. Y., Reinhold.

Jackson, D. S., and Bentley, J. P. (1960), *J. Biophys.*

Biochem. Cytol. 7, 37.
Jansson, E. H., and Weaver, E. S. (1964), Abstracts, 147th National Meeting of the American Chemical Society, Philadelphia, Pa., April, p 6H.
Johnston, J. P., and Ogston, A. G. (1946), *Trans. Faraday Soc. 42*, 789.
Kenchington, A. W., and Ward, A. G. (1954), *Biochem. J. 58*, 202.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Steinhardt, J., and Beychok, S. (1964), *in* The Proteins Composition, Structure, and Function, Vol. II, Neurath, H., Ed., New York, N. Y., Academic, Chapter 8.
Stevens, F. S., and Tristram, G. R. (1962), *Biochem. J. 83*, 245.
Tanford (1955), *in* Electrochemistry in Biology and Medicine, Shedlovsky, T., Ed., New York, N. Y., Wiley, Chapter 13.
Tanford, L. (1962), *Advan. Protein Chem. 17*, 69.

Purification and Some Properties of Tryptophanase from *Bacillus alvei**

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ABSTRACT: Tryptophanase was purified from cell-free extracts of *Bacillus alvei*. The enzyme was homogeneous by sedimentation and electrophoretic criteria, and was 3400-fold purified over the crude extract. Potassium or ammonium ions are required for enzyme activity; sodium ions are inhibitory at concentrations >0.1 M. At 80°, the enzyme is only slightly denatured within 10 min but at 100°, 90% of the activity is lost in 10 min. Heat stability is enhanced by pyridoxal phosphate, but not by pyridoxal; cysteine decreases the heat stability.

The Michaelis constant for the enzyme–tryptophan complex was 0.27 mM. The dissociation constants, kinetically determined, for competitive inhibitors were 3.23 mM phenylalanine, 0.107 mM anthranilic acid, and 0.71 mM kynurenine. The enzyme is apparently specific for L-tryptophan, and does not catalyze any chemical change in the above inhibitors. The amino acid composition was reported and differs from the composition of *Escherichia coli* tryptophanase. The turnover number of the enzyme is 1400 moles of indole formed/min per 100,000 g of enzyme. Rabbit antiserum prepared *vs.* pure tryptophanase from *B. alvei* forms a single precipitin band in gel diffusion plates with either pure enzyme or crude extract, but forms no precipitin band with purified enzyme from *E. coli*.

The enzymatic fission of tryptophan to indole, pyruvate, and ammonia is catalyzed by the enzyme tryptophanase. The stoichiometry of this reaction was first shown by Wood *et al.* (1947) who partially purified the enzyme from *Escherichia coli*. They also showed that the catalysis was pyridoxal phosphate dependent. Their enzyme preparation was free of serine and alanine deaminase activity, thus ruling out the possibility that the reaction was analogous to a reversal of the tryptophan synthetase reaction (Tatum and Bonner, 1944) or that the products of the reaction were indole and alanine (Baker and Happold, 1940).

Burns and DeMoss (1962) first isolated the enzyme in pure form from a tryptophan auxotroph of *E. coli* K-12. Their enzyme preparations were pure as judged by sedimentation and electrophoretic criteria. The protein they obtained was of a high molecular weight. A sedimentation coefficient of 9.0 S was obtained and a molecular weight of 490,000 was calculated from sedimentation equilibrium data. The enzyme was found to be unstable in either pure or crude form. Recently Newton *et al.* (1965) have crystallized tryptophanase from a mutant of *E. coli* B. They reported the molecular weight to be 281,000, a value which is probably more nearly correct than that previously estimated by Burns and DeMoss (1962). The enzyme has been found by Newton and Snell (1964) to catalyze the formation of tryptophan from indole and serine. Serine could be replaced by either cysteine or *S*-methylcysteine. The enzyme will catalyze a number of other α,β-elimination reactions analogous to the tryptophanase reaction.

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