Titration of Human Skin Collagen Extracted with Acid*

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ABSTRACT: Hydrogen ion titrations were used as a method to study the ionizable groups which may be involved in bonding in an apparently highly cross-linked form of soluble collagen. Titrations were done on the undenatured and the heat-denatured (40°, 30 min, pH 6.5) forms of a purified and physically characterized collagen which was extracted from human dermis with pH 1.5 citrate solution. Heat denaturation resulted in the unmasking of 7.5 carboxyl groups/10^5 g, 1.0 imidazole or α-amino group/10^5 g, and 6.5 ϵ-amino groups/10^5 g. The release of these groups was associated with loss of helical structure and separation of the protein into α and β components (α:β = 1:9).

The unmasking of these ionizable groups is thought to result from the disruption of interchain bonds involving these groups. Furthermore, after the heat denaturation, *not* all groups known to be present from amino acid analyses were titrated, indicating the presence of groups which remained masked. The quantitative values for these masked groups were: 5.0/10^5 g of carboxyl groups, 0–0.5/10^5 g of imidazole or α-amino groups, and 6/10^5 g of ϵ-amino groups. These masked groups are thought to be involved in interchain bonds between the polypeptide chains which make up the cross-linked β components.

The objective of this investigation was to study the titratable groups in a soluble collagen which is apparently highly cross-linked. Comparisons could then be made to data (Hartman and Bakerman, 1966) obtained from a soluble collagen which has relatively fewer cross-links. These comparisons may eventually be valuable in determining the chemical process of collagen maturation in tissue.

The soluble collagen was isolated from human skin with 0.15 M citrate at pH 1.5 following exhaustive extractions with the more conventional solvents (neutral salt and citrate buffer, pH 3–4). The low pH used in the extraction is different from that usually used to extract undenatured molecules; however, it has been shown by Burge and Hynes (1959) that this pH does not cause denaturation under comparable conditions. In fact, the isolated protein had the properties which are considered to be indicative of collagen in the undenatured state. These properties included high negative optical rotation, $[\alpha]_D^{20}$ −414°, and a single peak in analytical ultracentrifuge schlieren patterns. The changes in physical properties on heating to 40° were compatible with undenatured collagen; *i.e.*, optical rotation became less negative, $[\alpha]_D^{20}$ −115°, and two peaks were present in the ultracentrifuge patterns. This molecular species is of particular interest in that it yielded predominantly β components on heat denaturation (Bakerman and Hersh, 1964). This is in contrast to the salt-extracted collagen which yielded predominantly α components following similar treatment. Although the precise structure of this soluble fraction is not well understood, its titration provides a means of obtaining information concerning a species of collagen which contains a high proportion of the cross-linked β components.

Titration curves were obtained on the undenatured solubilized collagen and on the components which were obtained following heat denaturation. Differences between the kinds and numbers of groups titrated in the two states gave the type and numbers of groups unmasked. Further comparison of the possible number of titratable groups as determined by amino acid analyses with the number actually titrated after denaturation gave the groups which remained masked.

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 at least 1000 times its volume of triple-distilled water for 48 hr to remove water-soluble material.

The washed dermis was extracted with 0.15 M NaCl (1:4, tissue:solvent, v/v), pH 7.4, for 24 hr, and the supernatant fluid was removed following centrifugation at 59,000*g* for 1 hr. The extractions were repeated four to five times or until no visible precipitate could

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be obtained following dialysis of the supernatants against at least 1000 times its volume of 0.01 M disodium hydrogen phosphate for 48 hr. The remaining tissue was then placed in contact with 0.45 M sodium chloride and the above iterative procedure was repeated. Likewise, the procedure was carried out in the following order with: 1.0 and 2.0 M sodium chloride, 0.2 M citrate buffer (0.1 M citric acid and 0.1 M sodium citrate, pH 4.3), 0.5 M acetic acid. It should be noted that, following salt extractions and prior to acid buffer extraction, the residual salt in the tissue was removed by dialysis against at least 1000 times its volume of water.

The above conventional extraction procedures have been shown to extract undenatured collagen molecules in many different laboratories. Since lowering the pH appeared to be an important factor in increasing the yield of soluble undenatured collagens, it was speculated that even lower pH should extract additional undenatured collagen. On the basis of an article by Burge and Hynes (1959), it was evident that even pH 1.2 did not denature soluble collagen as long as the temperature was maintained below the denaturation temperature. Therefore, the following extraction procedure on the remaining tissue was carried out.

The tissue was placed in contact with 0.15 M citrate buffer (0.1 M citric acid and 0.05 M sodium citrate; 1:4, tissue:solvent, v/v), and the pH was reduced with stepwise addition of 2 M hydrochloric acid until the pH was 1.5. The supernatant fluid was collected after 24 hr by centrifugation and saved. The tissue was repeatedly extracted in the same manner with the citrate at pH 1.5, then centrifuged, and each of the supernatants was saved separately. With continual extraction, the yield gradually decreased until after about seven extractions no additional collagen could be precipitated following dialysis against the disodium hydrogen phosphate. Each of the supernatant fluids in citrate at pH 1.5 was dialyzed against at least 1000 times its volume of the 0.01 M disodium hydrogen phosphate for 48 hr to precipitate the collagen and then dialyzed against water.

The precipitates of the low pH extracted collagen were next dialyzed against 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 low pH extracted collagen were also dialyzed against formate buffer, pH 3.75, $\mu = 0.15$, for certain physical measurements which are detailed below. Extractions 4–6 of the low pH extracted collagen were used in the titration studies because these contained the highest percentage of β components and gave high yield.

Amino Acid Analyses. Complete amino acid analyses were done according to the method of Spackman *et al.* (1958). Cystine and cysteine were shown to be absent, and tyrosine was present in the amount of 2.4 residues/10^5 g. These results are indicative of a high degree of purity. Only the distribution of amino acids "characteristic" of collagen and of those residues which contain titratable reactive groups on side chains are presented (results in residues/10^5 g, mean residue weight 91.5 g): hydroxyproline, 99; proline, 137; glycine, 362; glutamic acid, 79.3; aspartic acid, 50.3; combined side-chain carboxyls, 129; amide nitrogen, 40; histidine, 6.4; lysine, 28.5; hydroxylysine, 7.3; combined side-chain amino groups, 35.8; arginine, 56.

Physical Characteristics. The physical characteristics of the low pH soluble collagen were determined in formate buffer and optical rotation was done in both formate buffer and 0.5 *m* KCl, *i.e.*, both in undenatured and denatured states. Denaturation of the soluble protein by heating to 40° for 30 min was done both in formate buffer and in 0.5 *m* KCl at pH 6.5.

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 rotor type An-E and temperatures of 20° for the undenatured collagen and 35° for the denatured collagen were used. With a path length of 30 mm 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. A value of 0.704 ml/g for $\bar{V}$ was determined by calculation from the amino acid analyses.

Optical rotation was measured with a Rudolph photoelectric polarimeter, Model 200, at the sodium D line 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 formate buffer and in the same solvent (0.5 *m* KCl) as that used in the titration.

Concentrations were determined by Conway (1957) diffusion techniques preceded by Kjeldahl digestion. 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 elsewhere (Hartman and Bakerman, 1966).

Summary

The physical characteristics of the low pH collagen used in the titration were as follows: *undenatured collagen:* sedimentation coefficient, $s^0_{20,w} = 3.15 \pm 0.15$ S; specific rotation, $[\alpha]^{20}_D$ 415 ± 30°; *denatured collagen:* specific rotation, $[\alpha]^{20}_D$ 115 ± 10°. The ultracentrifuge patterns obtained from the third extraction at low pH, corrected for the Johnston–Ogston (1946) effect of the denatured collagen, showed 10% α com-

ponent and 90% β component by weight. This preparation was used in the titration studies.

Titration Procedure. The equipment procedures, precautions, assumptions, and calculations are identical with those used in the titration of salt-extracted collagen (Hartman and Bakerman, 1966). These were based on the methodology described in detail by Tanford (1955, 1962) and Kenchington (1960).

The procedure for the titration was as follows. (1) Fresh aliquots of undenatured collagen solution were used for the acidic and basic titrations at 20°. (2) Each sample was titrated from a pH near seven to the respective extreme of pH. (3) The sample was then back-titrated to neutral pH with an equivalent amount of the opposite reagent. (4) The sample was then retitrated to determine reproducibility. (5) Samples were heat-denatured, and procedures 1–4 were repeated. A single titration from neutral to the pH extreme was extended over a 3-hr period. Optical rotation was measured after titrations of the (a) undenatured collagen and (b) heat-denatured collagen, to monitor the configurational status of the molecules.

Brief Discussion of Errors. The *absolute* error predicted for individual titration curves was calculated from the 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 1 group/10^5 g at near-neutral pH values. For reasons discussed elsewhere (Hartman and Bakerman, 1966) when the difference between two values at two points on similar titration curves is computed, the resultant error in these differences is lower than the absolute error at these two points. Thus, the error in the numbers of groups *unmasked* is substantially less than the absolute error.

The number of groups which *remain masked* is obtained by subtracting the number of groups titrated after denaturation from the number of groups known to be present by amino acid analyses. Since these quantities are obtained by methods in which the errors originate from different causes, the errors will not necessarily be in the same direction and cancellation may not occur on subtraction. Thus, errors in the final results will be of the same order of magnitude as the errors in the absolute values.

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 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 or cystine. Therefore, there was no need to assign an area of the titration curve to sulfhydryl groups. There were only 2.4 residues/10^5 g of tyrosine. These groups will titrate in the basic region of the curve with the ϵ-amino groups of lysine and hydroxylysine. Assuming these phenolic groups are titrated, then by subtracting the 2.4 phenolic groups from the number of groups titrated in the ϵ-amino pH range, the number of ϵ-amino groups is obtained. Further simplification of analysis is made possible by the paucity of α-amino and carboxyl end groups in collagen; there are no more than $1/10^5$ g of these groups and possibly even less (Stevens and Tristram, 1962; found $0.1/10^5$ α-amino groups in acetic acid extracted collagen). Lastly, guanidyl groups of arginine are usually found to titrate at pH values outside the range of our titration.

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, 2–3 mg/ml, reduced protein–protein interaction. In order to facilitate analysis, it was assumed that solubilized collagen is completely penetrable by aqueous hydrogen ion.

Results

Analysis of Titration Curves. The titrations in both the undenatured state and the denatured state were found to be reversible and reproducible. The experimental titration curves are given in Figure 1. The point of zero hydrogen ion binding was placed at the acid end point of each of the curves. All points at higher pH represent hydrogen ion dissociated/10^5 g of collagen.

Cannan (1942) presented criteria for the division of titration curves into regions representing the various ionizable groups. Cannan's criteria applied to the groups in our material are as follows: (a) pH 2.0–6.0, carboxyl groups;[1] (b) pH 6.0–8.5, α-amino groups and imidazole groups; (c) pH 8.5–11.5, phenolic groups and ϵ-amino groups.

The values of the respective ionizable groups obtained by applying these criteria to the titration curves of the undenatured protein and the heat-denatured protein are given in Table I. Optical rotation was measured following the titrations. For the undenatured collagen, no change was observed from the original value, $[\alpha]_D^{20}$ −415°, thus indicating that no detectable loss of helical structure had occurred during this titration procedure. Following heat denaturation (40°, 30 min, pH 6.5) optical rotation was again measured and found to be $[\alpha]_D^{20}$ −115°, indicating the loss of helical structure; ultracentrifugation confirmed the appearance of predominantly β component. Titration and retitration were then done on the denatured material. Optical rotation measured after the titrations indicated no measurable renaturation had occurred ($[\alpha]_D^{20}$ −115°).

The pK Observed. Comparisons are made to the p*K*

[1] The carboxyls which are titrated may 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 *et al.* (1963).

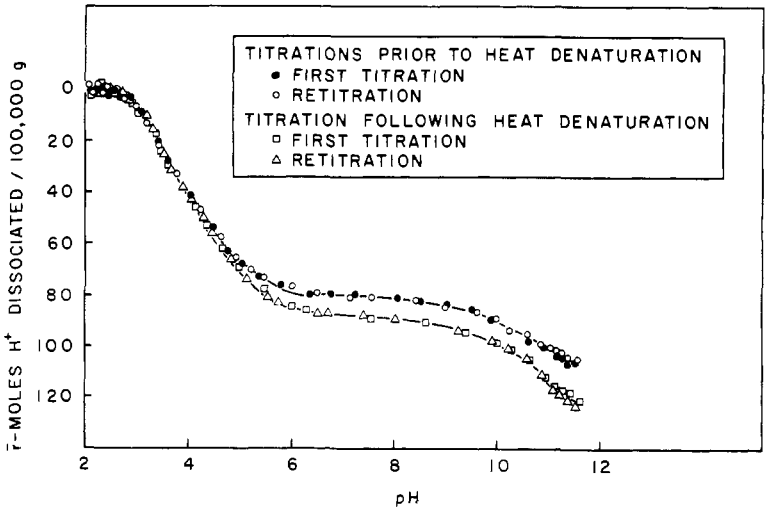

FIGURE 1: Titration curves for pH 1.5 citrate-extracted human skin collagen at 20° in KCl, $\mu = 0.5$.

TABLE I: Summary of Titration Data of Human Skin Collagen Extracted at Low pH.[a]

	Undenatured		Heat Denatured	
Group	Groups/ 10^5 g	pK_{obsd}	Groups/ 10^5 g	pK_{obsd}
Carboxyl	77	3.95	84.5	4.1
Imidazole and α-amino	5.5	7.1	6.5	6.8
Phenolic and ε-amino	24	10.5	32	10.6
Guanidyl	Could not be evaluated			

[a] Titrations at 20 ± 0.1°.

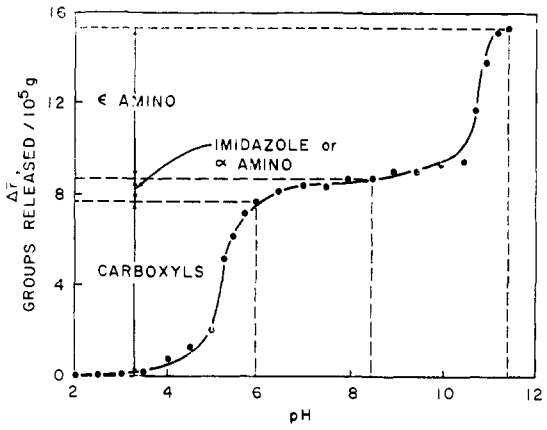

FIGURE 2: Groups released in moles/10^5 g of collagen.

of the salt-extracted protein (Hartman and Bakerman, 1966) and other proteins where appropriate. For the carboxyl region, the $pK_{obsd} = 3.95$ for the undenatured protein and 4.1 for the denatured protein. The pK_{obsd} for the denatured protein is identical with that of the salt-extracted protein ($pK_{obsd} = 4.1$).

For the imidazole groups, $pK_{obsd} = 7.1$ for the undenatured protein and 6.8 for the denatured protein. The range of these values encompasses the value found in the denatured form of the salt-extracted collagen ($pK_{obsd} = 6.9$).

For the ε-amino region, $pK_{obsd} = 10.5$ for the undenatured protein and 10.6 for the denatured protein. These values are slightly higher than the expected value; $pK_{int} = 10.4$ (Steinhardt and Beychok, 1964). The values observed, however, are close to those found in other proteins (metmyoglobin, $pK_{int} = 10.6$, zinc insulin, $pK_{int} = 10.5$; Steinhardt and Beychok, 1964).

It is of particular interest that the pK_{obsd} is markedly different from that found in salt-extracted collagen ($pK_{obsd} = 9.8$). This is probably due to differences in electrostatic and inductive forces in the two types of molecules, and could conceivably be an important factor in determining the relative strength of bonds which involve these groups in the two molecular species. The values of the pK observed are presented in Table I.

Discussion

The major objectives of this investigation were twofold. The first objective was to identify and quantitate those ionizable groups which were unmasked during heat denaturation. The second objective was to identify and quantitate those ionizable groups which *remain* masked *even after* heat denaturation.

Ionizable Groups Unmasked by Mild Heat Denaturation. These groups were measured directly by computing the differences between the titration curve of the undenatured collagen and the titration curve of

the denatured collagen. These differences are plotted against pH in Figure 2. The presence of a plateau region between the acidic and basic regions permits the very accurate identification of the groups. The total number of reactive groups assigned to carboxyl groups or ϵ-amino groups, respectively, will not be changed by more than 1.0 group as long as the end points of these groups are placed between pH 6.00 and 8.5.

The numbers of the various groups released by mild heat denaturation are given in Table II. The unmasking

TABLE II: Ionizable Groups Released by Mild Heat Denaturation.

Group	Groups Released/ 10^5 g
Carboxyl	7.5
Imidazole and α-amino	1.0
ϵ-Amino	6.5
Guanidyl	Could not be evaluated

of these groups was associated with a drop in optical rotation from $[\alpha]_D^{20}$ -410 to $[\alpha]_D^{20}$ $-115°$, indicating a loss of the helical configuration; ultracentrifugation confirmed separation of the molecules into predominantly β component.

Ionizable Groups Which Remain Masked Following Heat Denaturation. These groups cannot be measured directly, but may be computed indirectly by subtracting the numbers of the various ionizable groups which were titrated after denaturation from the respective numbers of groups which were determined by amino acid analyses. The difference between these two quantities will yield the number of groups which remain masked after heat denaturation.

With the exception of the carboxyl groups, this computation is straightforward. In the case of carboxyls, 84.5 groups/10^5 g were titrated and 129.6 side-chain carboxyls are known to be present by analysis. Thus 45 carboxyl groups remain masked after heat denaturation. Amino acid analyses showed, however, that 40 of these carboxyls are masked in the form of glutamide and aspartamide. Thus, only five of these carboxyl groups can be considered as possible participants in types of bonding other than the amides of glutamide and aspartamide.

The numbers of ionizable groups which remain masked following heat denaturation are given in Table III. It should be pointed out that considerably less confidence can be placed in the accuracy of the values of the numbers of groups remaining masked than in the values for the numbers of groups unmasked. In the

TABLE III: Ionizable Groups Remaining Masked Following Heat Denaturation.

Group	Amino Acid Anal./ 10^5 g	Groups Titrated/ 10^5 g	Groups Remaining Masked/ 10^5 g
Side chain carboxyl	129.6	84.5	45
Amide nitrogen	40.0		
Masked carboxyl groups other than amides			5
Imidazole	6.7	6.5	0–0.5
Phenolic	2.4		
ϵ-Amino	35.8		
Total	38.2	32	6

former case errors in the *absolute* values are not cancelled by subtraction as they are in the latter case (see Discussion of Errors).

Additional Considerations of the Titration Data. THE HEAT-LABILE TYPE OF BOND. The presence of bonds which were disrupted by heat was implied in the unmasking of ionizable groups after exposure to heat. The association of the release of these groups with the loss of helical structure and separation into its component chains tends to implicate them as possible participants in interchain cross-links. The fact that the carboxyl and ϵ-amino groups were released in approximately equimolar quantities (7.5 carboxyl *vs.* 6.5 ϵ-amino) may mean that both bonding sites were derived from the rupture of a single bond or a single cross-link.

Bonds similar to these were also present in salt-extracted collagen (Hartman and Bakerman, 1966) with the exception that the groups were also released by exposure of the protein to high pH, whereas, in the collagen presently under consideration, there was no detectable unmasking at either extreme of pH. It is unlikely in either case that covalent bonding (unless of extremely labile type) is responsible for masking of bonds which are released by such mild heat treatment. Very labile covalent or electrostatic bonding would, therefore, seem the most likely explanation for masking. From the experimental data, it is impossible to exclude masking as a result of aggregation. It should be emphasized that low protein concentrations were used in order to minimize this possibility.

THE HEAT-STABLE TYPE OF BOND. The ionizable groups which remain masked after exposure to heat (as evidenced by the incomplete titration of all the reactive groups known to be present by amino acid analyses) most probably participate in the interchain cross-links between the two α components which are thought to make up the β components.

It is seen (Table III) that approximately equimolar quantities of carboxyl bonding sites and ε-amino bonding sites *remain masked* after heat denaturation (5.0 carboxyl *vs.* 6.0 ε-amino). This observation leads us to conclude once again that the groups may be involved in pairs either in the form of a single bond or a single cross-link. Because of the difficulty in unmasking *these* groups (*i.e.*, they were never unmasked by our procedures) it would seem more likely that these groups *are* involved in covalent bonding. Although this conclusion is implied from these data, it cannot be proven until the cross-links between these groups are actually isolated. In this direction, Bornstein *et al.* (1966) have isolated a peptide chain derived from soluble collagens which contained a specific lysyl residue apparently participating in intramolecular interchain cross-linking. Bowes *et al.* (1965) reported that all the ε-amino groups of insoluble collagen are available to fluorodinitrobenzene under appropriate conditions; however, the conditions usually used in this reaction (40°, 1.5–24 hr) are more stringent than those used in our techniques of denaturation (40°, 30 min). Furthermore, other authors have reported much lower values for the availability of ε-amino groups by reaction with fluorodinitrobenzene.

The extent of collagen fold in the denatured state was measured by optical rotation at the same temperature as the titration. Titrations were done at 20° rather than at the higher temperature because of the experimental difficulties of controlling evaporation and because of the somewhat erratic behavior of the electrodes and automatic titrator at 40°. The titrations were carried out immediately following heat denaturation in order to reduce the influence of collagen refolding at the lower temperature. Furthermore, the value for the optical rotation, $[\alpha]_D^{20}$ −115°, was compatible with that reported by other workers on completely denatured material.

Low pH Extractable Collagen vs. Salt-Extractable Collagen. Comparisons of the data obtained from low pH extractable collagen can now be made to that obtained from salt-extracted human skin collagen (Hartman and Bakerman, 1966). Prior to heat denaturation, there were nearly twice as many groups unavailable to titration in low pH extractable collagen than in salt-extractable collagen. This might be expected since the former is thought to be more highly cross-linked. Following heat denaturation, approximately one-half of the masked groups in low pH extractable collagen remained masked whereas all masked groups were titratable in the salt-extracted molecules. This also might have been predicted since the low pH extractable collagen denatured into the cross-linked β components whereas the salt denatured into the single non-cross-linked α components. In all cases, the same number of carboxyl and ε-amino groups were either released or remained masked following heat denaturation.

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