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Studies on the Purification of Collagen

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Several methods of purifying collagen were studied. The effects of particular treatments on collagen were followed by nitrogen determinations, by electrometric titration analyses, and by electrophoretic measurements.

Treatment of the raw material with trypsin, as in the conventional method for the preparation of collagen, results in changes that lead to a degradation of the collagen in subsequent extractions. Treatment with dilute salt solution followed by trypsin produces a material of comparatively low isoelectic point (pH 5.5). Apparently soaking in dilute salt solution is not detrimental to collagen, and it is recommended that this type of extraction be substituted for the extraction with trypsin.

A technique for removing all but a negligible part of the mineral content from collagen is described, and a procedure is recommended for use in the preparation of ash-free gelatin.

A specification is proposed for purified collagen: (1) The water extract shall have a pH range of 6.0 to 7.0; (2) the isoelectric point (determined electrophoretically) shall be between pH 6.0 and 7.5; (3) the material shall have an ash content of less than 0.1 percent, total nitrogen content of 17.8 to 18.1 percent, and an amide nitrogen content (determined by hydrolyzing for 20 hours in 0.1 *N* hydrochloric acid at 90° C) of at least 3.8 percent, expressed as percentage of total nitrogen.

I. Introduction

As a preliminary to proposed work on the chemistry of collagen, it is considered necessary to reexamine the methods that have been used in the past for the preparation of a purified material. The ultimate objective of a process for the purification of collagen is the production of a pure, uniform, readily reproducible material in virtually the same physical and chemical state in which it exists in the raw hide.

The chief source of collagen is raw cattle hides. Coexistent with the collagen in the hide are small quantities of extraneous materials as outlined in figure 1. The problem is to effect a separation of these materials with the least possible alteration of the chemical structure of the collagen.

In 1936 Highberger [1] [1] proposed a method of purification that has been generally used in this country. The method includes tryptic digestion of fresh corium under carefully controlled conditions, removal of fats with organic solvents, extraction with half-saturated calcium hydroxide solution, and deliming with acetic acid.

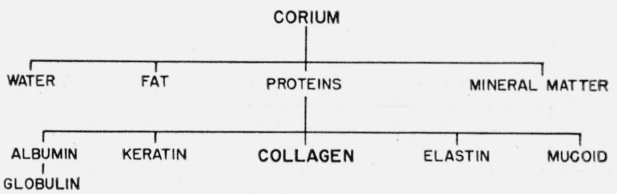

FIGURE 1. *Outline of the materials coexistent with collagen in the hide corium.*

The use of trypsin has been the subject of much discussion. The work of Thomas and Seymour-Jones [2] and that of Merrill and Fleming [3] showed that hide powder, obtained from cowhide that was strongly limed for dehairing purposes, was digested by trypsin. However, Marriott [4] showed that collagen-containing material could be extracted with a trypsin solution under carefully controlled conditions with practically no adverse

[1] Figures in brackets indicate the literature references at the end of this paper.

effect on the collagen if the collagen-containing material had not been previously subjected to the action of reagents likely to cause some alteration. Recently it has been reported that British investigators [5] have elected to omit the trypsin treatment and the extraction with half-saturated calcium hydroxide solution.

The present research was designed primarily to determine the effect on collagen of the various reagents used during its purification, and special effort was made to ascertain the effect of trypsin. The studies included determinations of amide nitrogen, soluble nitrogen, and total nitrogen, electrometric titration analyses, and electrophoretic measurements.

II. Experimental Material

The collagen used in this work was prepared in the leather laboratory of this Bureau from steer hide obtained from the Beltsville Agricultural Research Station. The hair was closely clipped within an hour or two after the animal's death. The hide was cut into strips 4 by 60 in., parallel to the backbone and portioned into four groups for the separate purification methods.

The preliminary treatments of purification varied between groups and are given in figure 2.

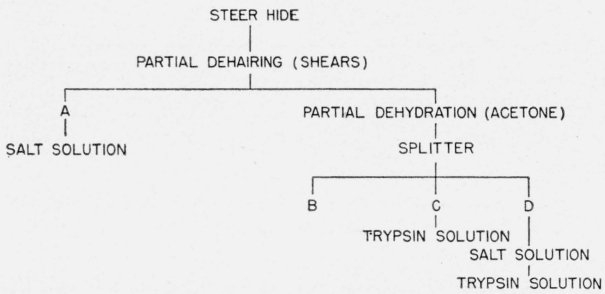

FIGURE 2. *Schematic diagram of the initial steps of the purification methods.*

After this preliminary treatment, all portions were subjected to the following treatment: Acetone, alcohol, lime solution, acetic acid, water, alcohol, and ethyl ether.

One portion (A) was placed immediately in a 5-percent sodium chloride solution, which had been chilled to about 6° C. The salt liquor was drained off daily, and fresh solution was added. After a month of such treatment, during which time the temperature of the salt solution was maintained at 6° C, the epidermal layer could be scraped off with a knife. The fatty layer was removed by the splitting machine. Extraction with trypsin was not included in the preliminary treatment of this portion of the hide.

The remaining three portions (B, C, and D) of the hide were partially dehydrated by treatment in acetone overnight to facilitate splitting off the epidermal and fatty layers in the splitting machine. Batch B received no other preliminary treatment. Batch C was treated with trypsin according to the method suggested by Highberger [1]. The third portion of this subdivision of the hide, D, was subjected to an extraction with dilute salt solution in a manner similar to that described for batch A and was then treated with trypsin.

The principal steps of the purification scheme were the same for all batches of material and included the following treatments: acetone and alcohol extraction to remove fats; extraction with lime solution to remove mucoid protein; treatment with acetic acid to delime; washing with water; and dehydrating first in alcohol and finally in ethyl ether.

Collagen from a second steer hide was purified according to the method suggested by Highberger [1]. Specimens from each step of the process were used for electrophoretic measurements so that changes in the surface characteristics of the collagen caused by each treatment could be determined.

Two samples of hide collagen that had been prepared by the method of Highberger [1] several years previous to this work were also tested. In addition, two sample that had been prepared at Cincinnati University 8 years ago were compared with the collagen prepared in these experiments. One of the samples had been treated with trypsin solution, and the other had been treated with trypsin solution and lime solution.

Small portions of collagen were prepared from the bone and tendon of a steer by the method of Beek [6]. The process included the necessary acid extraction to remove the calcium phosphate from the bone. The treatment with trypsin was omitted in the preparation of one sample of the bone collagen.

The hide powder used in this work was the ALCA Standard Official Hide Powder Lot No. 21.

III. Methods and Results

Several methods of analysis were used to follow the specific effect on collagen of the various purification procedures. These methods included determinations of nitrogen, and of acid- and base-combining capacities, and electrophoretic measurements.

1. Treatment With Trypsin Solution

The extent of the action of the trypsin solution on collagen was estimated from the amounts of nitrogenous material dissolved. In each treatment 4 ml of a 1-percent trypsin solution was added per gram of moist corium. The corium covered with this solution was incubated at a temperature of 35° C for 20 hours. The supernatant liquid was then drawn off and 200-ml aliquots were retained for Kjeldahl nitrogen determinations. After rinsing the corium in distilled water, the process was repeated a second and third time.

The nitrogenous material in solution following each trypsin extraction is composed of that introduced by the water solubilization of albumins, globulins, and to some extent mucoid, and that produced by the action of the enzyme on the collagen material. This latter portion is, for the most part, the result of the specific solubility effect of trypsin on elastin [7]. The results are shown in figure 3. The ordinate of the figure represents the loss in nitrogen per 50 g of collagen for each extraction. The points along the abscissa represent the successive treatments of 20 hr each. In all except one instance the curves level off toward a small constant value after three extractions. The tendency for these nitrogen values to approach a constant is in agreement with the results obtained by Highberger [1] that the maximum effect of trypsin on elastin takes place during the first three extractions. The results with bone collagen indicate that degradation of the collagen becomes significant after the third extraction with trypsin. Apparently the acid treatment included in the preliminary steps of the purification of the bone collagen has made the material more susceptible to reaction with trypsin.

Highberger showed that when trypsin is deactivated the main portion of soluble nitrogenous material in the first two extractions does not arise from enzyme activity but is due to the presence of soluble protein. This would probably account

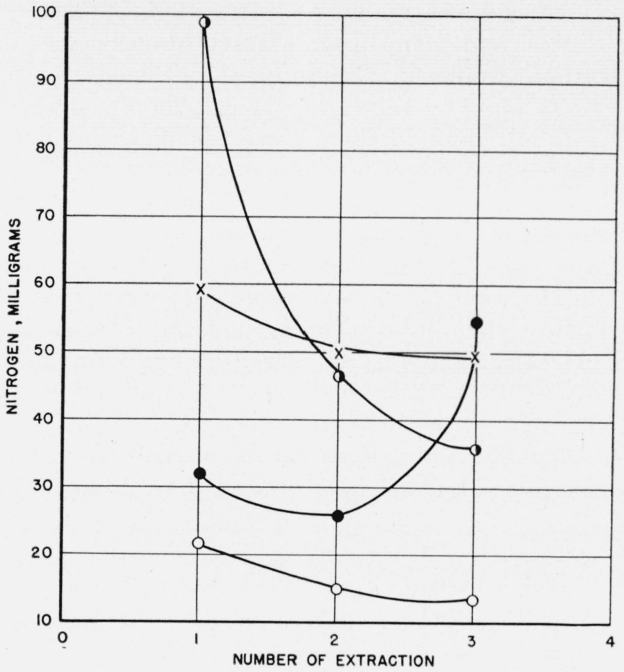

FIGURE 3. *Effect of preliminary treatment of collagen-containing materials on the nitrogenous compounds solubilized in subsequent trypsin extractions.*

The nitrogen values are per 50 g of collagen. ◑, Hide collagen (C), no previous treatment; ○, hide collagen (D), salt treated; ×, tendon collagen, washed in water; ●, bone collagen, acid treated.

for the comparatively high nitrogen result for the portion of corium C, on the first of the three extractions with trypsin. (See fig. 3.) Batch C had received no washing prior to the treatment with trypsin, whereas the other samples had been washed in aqueous solutions that solubilized some of the nitrogen-containing material.

2. Treatment with Calcium Hydroxide Solution

The extent of the action of half-saturated lime solution on collagen was followed by a series of tests similar to those used for studying the effect of trypsin. In each treatment 10 ml of the calcium hydroxide solution was added per gram of moist corium. The corium-lime solution mixture was agitated on a shaker at room temperature for 1 hr. The supernatant liquid was then drained off and 200-ml aliquots were retained for the Kjeldahl nitrogen determinations. The process was repeated a second and third time.

The purpose of this treatment is to remove the mucoid protein. McLaughlin and Theis [8] found 0.45 percent (calculated on dry basis) of this material in the corium of steer hide. This substance,

which contains about 2.4 percent of sulfur and 12.4 percent of nitrogen, exhibits properties of a carbohydrate as well as those of a protein.

The absolute values for the soluble nitrogenous materials given in figure 4 are not important, but the relative values for different collagens seem to have some significance. The four highest nitrogen values in the first extraction period are from those collagens that had been treated with trypsin. In the sample of bone collagen treated with trypsin, the soluble nitrogenous material from the first calcium hydroxide extraction was four times greater than that found in the sample of bone collagen not treated with trypsin.

A similar comparison can be made of two hide collagen samples, the purification of which included a treatment with dilute salt solution. The ratio of the nitrogen content of the exhaust liquors from the initial lime extraction of the sample treated with trypsin and salt solution and that treated with trypsin alone is 3:1. A comparison of the hide collagen samples that had no salt treatment and differed only in the trypsin treatment shows the same trend. The exhaust liquor from the first lime extraction of the sample previously treated with trypsin has one and a half times the nitrogen content of the exhaust liquor from the sample that had not been treated with trypsin. Apparently the treatment with trypsin changes the materials in some manner so that they are more easily degraded in subsequent treatment with lime water.

It should be pointed out that the material least affected by this lime extraction is the collagen which was leached in the 5-percent sodium chloride solution and which was not treated with trypsin.

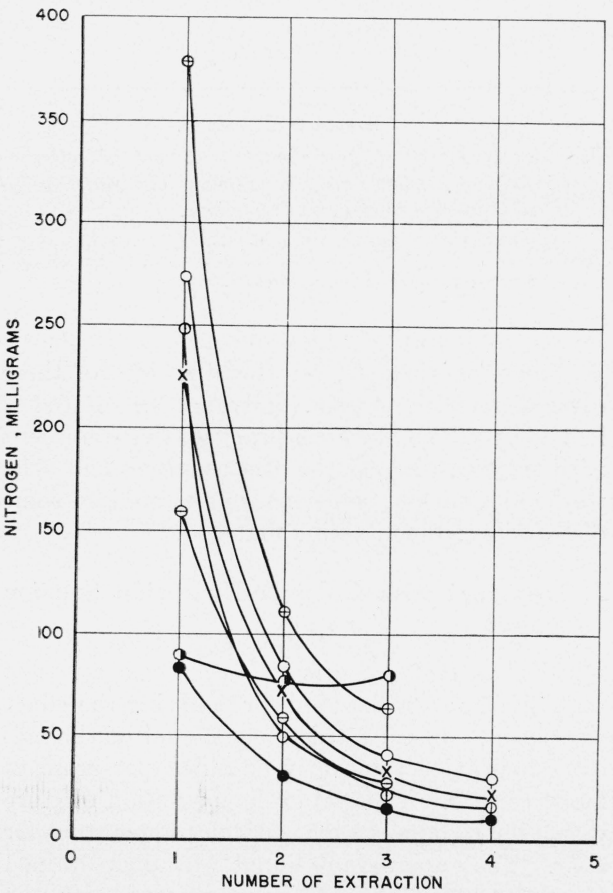

FIGURE 4. *Effect of previous treatment of collagen-containing materials on the nitrogenous compounds solubilized in subsequent calcium hydroxide extractions.*

The nitrogen values are per 100 g of collagen. ⊖, Hide collagen, no previous treatment; ○, hide collagen, treated with salt and trypsin; ◫, hide collagen, treated with trypsin; ●, hide collagen, treated with salt; ×, tendon collagen, treated with trypsin; ⊕, bone collagen, treated with acid and trypsin; ◑, bone collagen, treated with acid.

3. Determination of Total Nitrogen and Amide Nitrogen

Total nitrogen in the collagen samples was determined by the standard Kjeldahl method. The amide nitrogen contents of the collagens were determined by hydrolyzing in 0.6 *N* sulfuric acid for 3 weeks at 60° C and in 0.1 *N* hydrochloric acid for 20 hrs at 90° C. In both methods the solutions were neutralized and ammonia distilled over in the presence of an excess of magnesium oxide. As the amount of ammonia obtained varies with the conditions of hydrolysis and distillation [9], the results have no absolute significance. However, the relative differences between the results obtained from different samples under the same conditions of hydrolysis and distillation give a measure of the effects of the purification treatments on the amide nitrogen content.

In table 1 the amide values of columns 4 and 5 are for hydrolyses carried out at 60° and 90° C, respectively. By either method it is apparent that the amide value for the hide collagen that was extracted in the dilute salt solution and then treated with trypsin is approximately 0.25 percent lower than that for the other three hide collagens. The other hide collagens have approximately the same concentration of amide nitrogen. These results indicate that the trypsin treatment, when not preceded by soaking in dilute salt

solution, did not attack the amide linkage. However, the combination of the action of the dilute salt solution and the trypsin appears to have lowered the amide nitrogen.

TABLE 1. *Effect of various treatments on nitrogen values of collagen*

Sample	Source	Treatment	Amide nitrogen as percentage of total nitrogen hydrolyzed at—		Total nitrogen
			60° C	90° C	
					Percent
1	Hide	Blank	3.55	4.27	17.75
2	do	Trypsin	3.54	4.34	17.81
3	do	Salt solution	3.50	4.23	17.89
4	do	Salt solution, trypsin.	3.25	4.04	17.87
5	Bone	Acid	2.36	2.99	17.76
6	do	Acid, trypsin	2.22	2.94	18.00
7	Hide powder	Lime, trypsin	2.15	2.46	17.41

Table 1 shows that the amide nitrogen content of the hide collagen samples is considerably higher than that of the bone collagen or hide powder. The bone collagen was subjected to a long acid hydrolysis, and the hide powder to a severe liming process. In either treatment amide bonds would have been attacked and the nitrogen contents lowered.

The nitrogen content of collagen (expressed as total nitrogen) does not seem to be definitely related to the method of preparation. Although the bone collagen sample treated with trypsin had about 1.2 percent less amide nitrogen than the hide collagens, its total nitrogen content was was greatest of the group. In neither the bone collagen nor the hide powder was the loss in amide nitrogen apparent in total nitrogen content. This might be due to removal, during purification of collagen, of proteins with comparatively low nitrogen contents, a process which would, in effect, increase the percentage of nitrogen in the final product.

4. Determinations of Acid and Base Combining Capacities

Combination of acid and base with the several collagen preparations was determined in potassium chloride solutions of constant ionic strength. The volume and normality of potassium chloride were varied with each acid or base concentration to maintain an ionic strength of 0.1. To 1 g of protein in a glass-stoppered bottle was added 100 ml of a solution having a known concentration of acid or base and a known concentration of potassium chloride. The equilibrium pH was determined with a glass electrode after 24 hr. For each bottle containing collagen, a bottle was prepared containing only the 100 ml of the aqueous medium, and then the acid or base bound by the protein was determined by difference. Since large changes in acid (base) content produce only small changes in the pH of extremely acid (basic) solutions, the maximum acid (base) binding values are subject to error.

The titration results are shown in figure 5. Maximum acid-binding values lie between 0.84 milliequivalent per gram for hide powder and 0.89 milliequivalent per gram for the collagen that had been treated with dilute salt solution but not with trypsin.

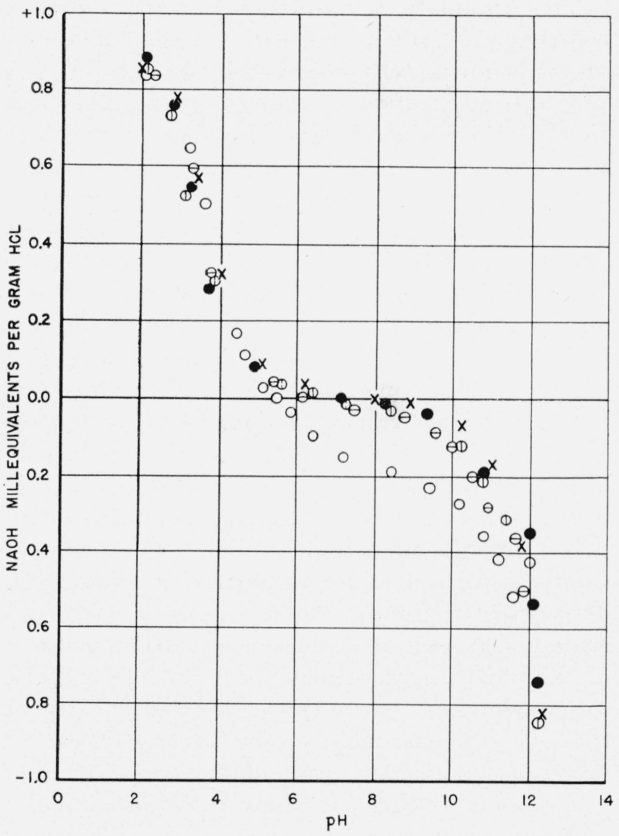

FIGURE 5. *Comparison of the acid-base titration curves of the various hide collagens and hide powder.*

○, Commercial hide powder; ⊖, collagen, trypsin and salt extracted; ⊙, collagen, no salt or trypsin treatment; ×, collagen, extracted with trypsin; ●, collagen, extracted with salt.

The collagen curves show a decided similarity regardless of method of preparation. The material that had been exposed to dilute salt solution and then to trypsin tended to have a more acidic nature than the other hide collagens. This is evidenced by its isoionic point of pH 6.2. The isoionic point is defined as the point at which dissociable groups of a substance combine equally and only with the hydrogen and hydroxyl ions [10]. Within the pH range 5.0 to 12.0 the curve for the hide powder indicates much greater base binding than that exhibited by any of the collagens. Between pH 7.0 to 9.0 this average difference is 0.17 milliequivalents. This may be accounted for by the difference in the average amide nitrogen content of the collagen samples and that of hide powder (0.16 milliequivalents).

5. Electrophoretic Analysis

The Abramson horizontal, microelectrophoresis cell that was used to make the mobility measurements is adequately described by Moyer [11]. A practical application of the cell in the investigation of wool protein is given by Harris [12]. The microscope was used with an 8-mm objective and a 20× eyepiece. The buffer mixtures were as follows: pH 2.4 to 3.6, potassium chloride-hydrochloric acid; pH 4.0 to 5.6, sodium acetate-acetic acid; pH 6.0 to 7.8 disodium phosphate-dihydrogen phosphate; pH 8.0 to 9.2, boric acid-borax, pH 10.0 sodium hydroxide-boric acid-potassium chloride. The ionic strength of all buffers was 0.005. The pH values were measured with glass electrodes and referred to 0.05 *M* potassium acid phthalate (pH 4.01), and to 0.01 *M* borax (pH 9.18). The specific resistance of solutions introduced into the cell was determined with a pipette conductivity cell and a conductivity bridge [11]. Two or three groups of measurements, each consisting of at least 10 measurements of the velocity of the particles, were made at each selected pH. When mobilities were very low, the velocity of the particles was measured in layers adjacent to the stationary layer, and mobility was evaluated by graphical integration of the curve obtained by plotting velocity against depth [13]. Sufficiently stable suspensions for the measurements were prepared with a Waring Blendor.

The mobility measurements were made in a room at approximately 23° C. All values were corrected to 25° C by applying a factor of 2 percent per degree [11].

As a check on the electrophoretic technique to be used, determinations were made of the mobilities of collagen previously prepared at this Bureau and of that sent to the Bureau from the University of Cincinnati. The results of these measurements are shown in figure 6. The isoelectric points determined by this work agree very well with those found by Beek and Sookne [14] and by Highberger [15] on these same materials.

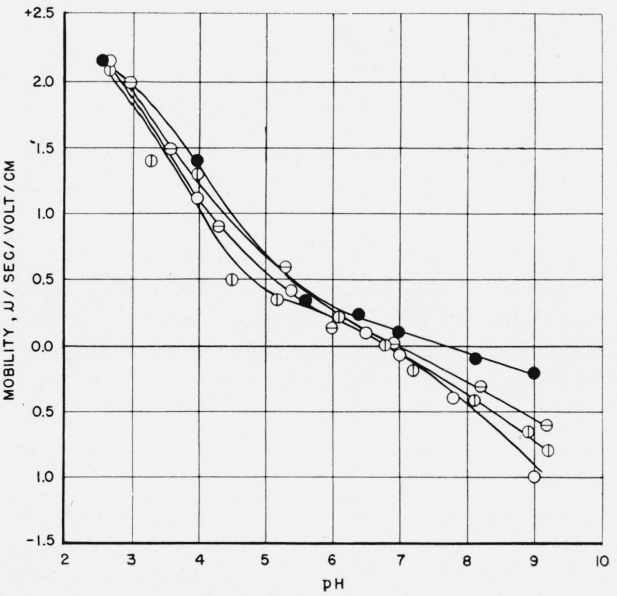

FIGURE 6. *Comparison of the* pH-*mobility curves for some hide collagens in storage for several years.*

⊖, Purified in the leather laboratory of this Bureau, Highberger procedure; ⊕, purified in the leather laboratory of this Bureau, Highberger procedure; ●, purified in another laboratory, Highberger procedure; ○, purified in another laboratory, Highberger method with elimination of lime treatment.

In figure 7 the mobility-pH curves are plotted for the hide collagen samples prepared in a manner discussed previously in the text. The isoelectric point (pH 5.5) of the material extracted with dilute salt solution and trypsin is lowest of this group.

The very close agreement in isoelectric point (6.4, 6.5, 6.4) of the other hide collagen preparations indicates that either trypsin or dilute salt solution, if used alone, will not seriously alter the surface characteristics of the product. However, a combination of these two extractions in preparation of collagen has produced a more acidic surface than would seem desirable in undegraded material.

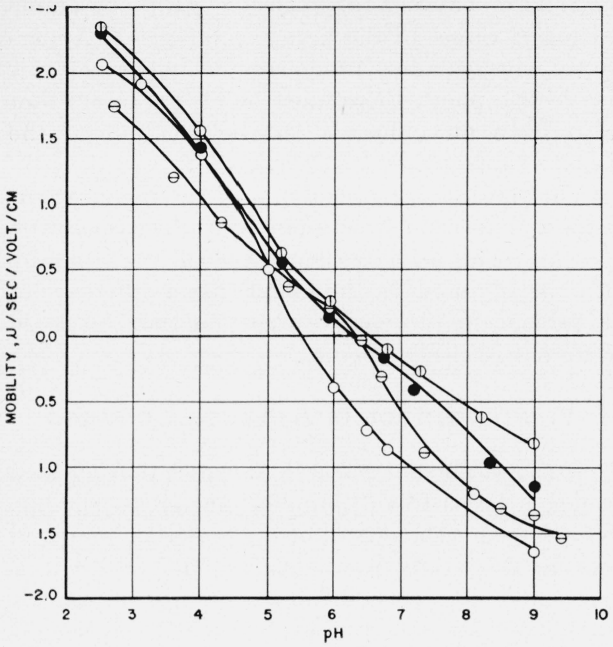

FIGURE 7. *Effect of method of purification on* pH-*mobility curve of collagen.*

○, Extracted with salt and trypsin; ◑, extracted with trypsin; ●, extracted with salt; ⊖, no extraction with salt or trypsin.

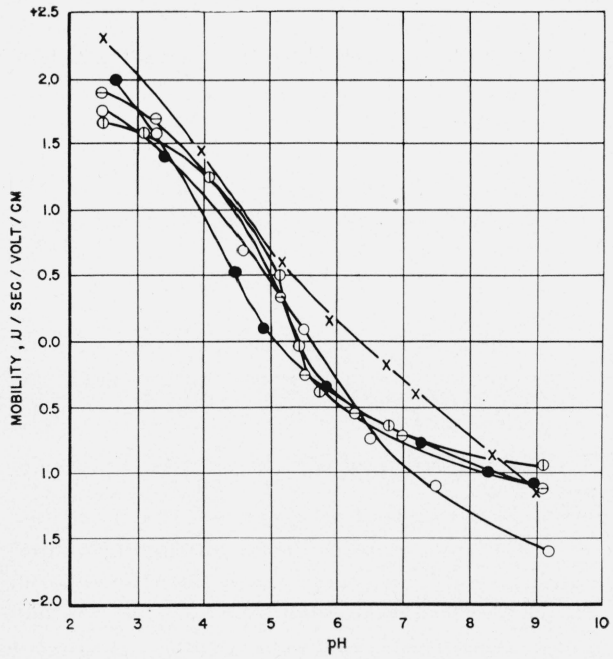

FIGURE 8. *Comparison of the* pH-*mobility curves of hide collagen with those of hide powder, bone, and tendon collagen.*

○, Tendon collagen; ◑, bone collagen, treated with trypsin; ⊖, bone collagen; ●, hide powder; ×, hide collagen.

The curves in figure 8 show the mobilities as a function of pH for collagen from hide, tendon, and bone, and for hide powder. The hide collagen is isoelectric about one pH unit higher than the other collagens. This is probably due to the drastic hydrolysis to which these materials were subjected during purification. Beek and Sookne [14] have suggested that the decrease in amide nitrogen content is accompanied by an increase in the number of free carboxyl groups, the net result of which is to shift the isoelectric point to a more acid region. The results obtained with the two bone collagen samples, one of which was treated with trypsin, are similar. Since the materials are isoelectric at the same pH value, the extraction with trypsin would seem to have had little effect on the characteristics of the collagen. However, results discussed earlier in this paper [2] indicate that trypsin has a deterimental effect on bone collagen previously subjected to prolonged treatment with acid. It can only be assumed that any degradation that occurs during the extraction with trypsin has no effect on the surface charge of the finished product.

Figure 9 shows the mobility curves of specimens of collagen taken from the material at certain steps of one of the purification processes. (See sec. II.) The isoelectric point of the raw, unpurified collagen is pH 5.8. Extraction with trypsin has not shifted the isoelectric point but has made the material more reactive, as evidenced by the

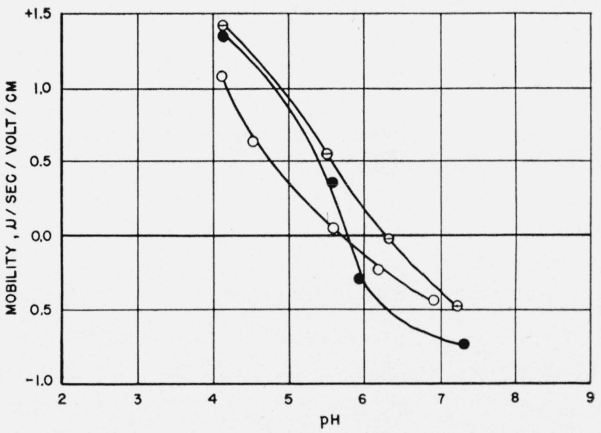

FIGURE 9. *The* pH-*mobility curves of collagen determined at various stages of the purification process.*

○, Raw collagen; ●, collagen treated with trypsin; ⊖, collagen subjected to complete purification.

[2] See discussion of nitrogen analyses made on trypsin extracts.

steeper slope of the curve on either side of the isoelectric point. The effect of the calcium hydroxide treatment has been to raise the isoelectric point. This shift in isoelectric point to a more alkaline region is the same sort of change shown in figure 6 when the unlimed material was isoelectric at pH 6.9, the limed material at 7.7. This shift may be accounted for by either of two reasons. If the mucoid removed by this extraction with half-saturated solution of lime is isoelectric at a pH lower than collagen, then its removal would raise the isoelectric point of the purified collagen product. Hesselvik [16] has made electrophoretic measurements on a number of mucoid samples and has shown that the isoelectric points of the materials varied from pH 3.0 to 4.5.

A second explanation, and one the authors believe to be nearer correct, is that some combination between carboxyl groups of the collagen and calcium atoms occurs during the lime extraction. Any tendency to inactivate the carboxyl groups would raise the isoelectric point. Presumably if all the calcium picked up by the collagen were not washed out in deashing the product, the isoelectric point of the product would be changed toward a more alkaline pH.

IV. Amide Nitrogen Content and Isoelectric Point

Previous investigators [14] have indicated that the isoelectric point and amide nitrogen content of collagen were related. In table 2 the amide content and isoelectric point are listed for several collagen samples. The two collagens of lowest amide nitrogen content have the lowest isoelectric points. However, one sample having about 1 percent greater amide nitrogen has an isoelectric point in the same region. There appears to be no correlation between the isoelectric point and amide nitrogen values of the first five samples. Apparently whereas a low amide content indicates a low isoelectric point, there must be other factors that also have an influence on the location of the isoelectric point.

TABLE 2. *Correlation of nitrogen values with isoelectric points*

Sample	Source	Amide nitrogen	Total nitrogen	Isoelectric point
		Percent	*Percent*	
1	Steer hide	3.93	17.84	7.7
2	do	4.14	17.97	7.0
3	do	3.83	17.38	6.9
4	do	4.34	17.81	6.5
5	do	4.27	17.75	6.4
6	do	4.01	17.71	5.5
7	Bone	2.94	18.00	5.4
8	Hide powder	2.46	17.41	5.1

The total nitrogen contents of the preparations appear to bear no direct relation to their isoelectric points. This same result with wool was noted by Harris [12] in 1932, who found that a difference of 1 percent in nitrogen content did not result in different isoelectric points.

V. Preparation of Ash-Free Collagen

It is desirable for research purposes that purified collagen be as free of mineral matter as possible. The average ash content of the materials prepared during this study was 0.06 percent. Since it is preferable in many instances to use a product with even less ash content, a method was sought that could remove the bulk of the retained mineral matter. The collagen was leached in frequent changes of distilled water kept at 6° C. The bulk of the leach water was ejected by pressing at 5,000 lb/in.^{2} in a Carver press. The conductivity and pH were determined for each extract. The product was considered as free of mineral matter as this technique would permit when after 4 days of contact with distilled water the conductivity of the extract was 5×10^{-6} mho and the pH was 6.5 (conductivity of distilled water 1.5×10^{-6} mho, pH 5.6 to 6.0). Spectrographic analysis of the dried collagen revealed 50 parts per million of copper and of iron, 20 parts of lead and of calcium, and 10 parts of tin and of chromium.

A procedure of the type outlined above would yield a collagen from which ash-free gelatin might be derived for dielectric and other fundamental studies.

VI. Specifications for Purified Collagen

It is believed that a specification for purified collagen might be of value in furthering fundamental research on collagen. It must be stressed that these proposed requirements only satisfy minimum standards and considerable tolerance is intended. The pH and ash content of a product are easily determined. With a minimum of effort the ash content can be kept below 0.1 percent and the pH between 6.0 to 7.0. The isoelectric point

of a purified collagen should not be less than pH 6.0 as isoelectric values below this indicate degradation, and not more than 7.5 since higher values indicate combined metals or degradation [17]. Total nitrogen content of the product should be between 17.8 and 18.1 percent. Amide nitrogen, expressed as percentage of total nitrogen and determined by hydrolyzing for 20 hr in 0.1 *N* hydrochloric acid, should not be less than 3.8 percent.

VII. Conclusion

Even though the surface characteristics of collagen (as determined by electrophoretic measurements) do not seem to be impaired by an extraction with trypsin, there is strong evidence that trypsin modified the material in such a manner that subsequent treatments result in degradation of the product. The large differences observed in the values for the soluble nitrogenous material in the lime extractions for the trypsin- and non-trypsin-treated collagens are evidence of this degradation. It was found also that in preparing bone collagen the nitrogen content of a third trypsin extract was double that of the first, and this increase in nitrogen could only arise by degradation of the material.

Although the dilute salt solution in combination with the trypsin has been shown to be detrimental to collagen, no evidence was observed that the use of the 5-percent sodium chloride solution alone was harmful. In view of the fact that the main portion of elastin is removed mechanically by splitting off the grain layer [18, 19] and since the salt solution removes the bulk of extraneous material without damage to the collagen, it is recommended that soaking in salt solution be used in preference to treatment with trypsin in the purification of collagen. Since it has been observed that a material extracted with dilute salt solution has not been degraded by a subsequent lime extraction, the use of a half-saturated calcium hydroxide solution would then be justified to remove the mucoid.

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