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COMBINATION OF HYDROCHLORIC ACID AND SODIUM HYDROXIDE WITH HIDE, TENDON, AND BONE COLLAGEN

By John Beek, Jr.

ABSTRACT

The amounts of hydrochloric acid and sodium hydroxide combining with three different samples of collagen were measured. The samples of collagen were prepared from the tissues of hide, tendon, and bone. The three materials combine with substantially the same amounts of acid, but the bone collagen combines with a larger amount of base than do the other two. This difference is explained by the loss of amide nitrogen during the preliminary treatment of the bone collagen, which indicates that the collagenous substances of the three natural tissues are identical.

CONTENTS

I. INTRODUCTION

An investigation of the nature of quinone tanning is in progress in this laboratory. As a part of the program of this investigation, some of the chemical properties of collagens from different tissues are being compared, with the object of obtaining evidence bearing on the question of the chemical individuality of white fibrous connective tissue. It is also proposed to compare the acidic and basic properties of collagen and quinone-tanned leather. This paper is a report of the work on the acidic and basic properties of samples of collagen prepared from the hide, tendons, and bones of a steer.

The reactions of collagen and gelatin with acids and bases have been studied by a large number of workers. A survey of the literature led to the conclusion that the best basis for comparing the amounts of acid or base combining with different samples of collagen would be obtained by measuring the quantity of acid or base taken up from an aqueous solution containing a high concentration of salt. Theoretically the use of a nonaqueous solvent or a gaseous acid or base would give more accurate results, but the inconvenience of making the corresponding measurements was considered prohibitive. For

example, the difficulty of eliminating water from such a system is considerable, and yet if any significant quantity of water is present the measurement becomes inaccurate.

II. THEORY OF THE MEASUREMENT

There are four conspicuous difficulties in the interpretation of the change in the concentration of an aqueous solution of an acid or base, brought about by the introduction of an insoluble protein.

First, there is the essential weakness of the measurement of adsorption from solution, resulting from the relative nature of the measurement. The only quantity that can be determined is a function of the amounts of the two components adsorbed and the equilibrium concentration of the solution. A third component may be added to the solution and used as a reference substance in calculating the amount of adsorption, but the calculation still depends on the assumption that the new substance is not adsorbed, and there is the further disadvantage, for the general case, that the nature of the system is changed. However, in the case of the system collagen-hydrochloric acid-sodium chloride-water, with the acid dilute and the salt approaching saturation, it has been shown [1] [1] that the amount of adsorption calculated with reference to the water is in agreement with that calculated with reference to the salt. Here no disadvantage arises from the presence of the salt, as it could not be expected to change the combining weight of the collagen.

Second, there is the uncertainty arising from the fact that a considerable quantity of solution passes into the protein phase. As the ratio of uncombined acid or base to uncombined water can not be directly determined in this part of the solution, any calculation of the total amount of uncombined acid or base in the system must depend on some assumption about the activity coefficients of the ions in the protein phase. For instance, in the system given in the preceding paragraph, the calculation of the amount of acid removed from solution, using the salt as reference substance, depends not only on the assumption that no salt is removed from the solution, but also on the assumption that the fractional change in the activity coefficient on passing from one phase to the other is the same for acid and for salt.

Third, there is the error introduced by the change of a part of the insoluble protein to a soluble protein, through the action of the acid or base. In the case of collagen, this effect has especial importance when alkaline solutions are used. In order to correct for the effect of the dissolved matter, it is necessary to make sure in the titration that all the acid or base in the solution is neutralized, whether or not it was combined with dissolved protein in the solution at equilibrium. Then, to calculate the specific adsorption for the solid protein, it is only necessary to consider the distribution of the total acid or base in the system between the part in solution (whether combined or not) and the part combined with the remaining solid protein. Of course, if the protein is completely dispersed, the calculated adsorption becomes indeterminate and the method fails.

The effectiveness of this method was tested experimentally by carrying out four measurements of the adsorption of sodium hydroxide by hide collagen in solutions about 0.08 molal in base, allowing the systems to stand for periods of 1, 2, 4, and 8 days. In this way the

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

fraction of the total nitrogen in solution was varied from 11 to 26 percent. The difference between the extreme values of the calculated adsorption was less than 4 percent, and the order of the determinations with reference to the amount of adsorption was not the same as the order with reference to the amount of collagen dissolved. These results indicate that this method of correcting for the effect of the dissolved matter is satisfactory.

Fourth, there is the problem of determining the amount of acid or base that is introduced as an impurity with the protein. The custom of experimenters in this field has been to dispose of the question by assuming either that the material as prepared contains no acid or base, or that the isoelectric point of the protein, as indicated by measurements of its electrophoretic mobility, corresponds to the condition in which the amounts of acid and base combined are equivalent. In the present work the amount of acid present in the collagen was measured directly. This was possible because the only acidic or basic substances used in processing [5] the collagen were calcium carbonate, calcium hydroxide and hydrochloric acid, so that the difference between the amounts of chloride and calcium present (expressed in equivalents) gave the acid content directly.

The assumption that the neutral point and the electrophoretically determined isoelectric point of collagen are identical was tested by putting a 5-g sample of hide collagen in 100 ml of water containing just enough sodium hydroxide to neutralize the acid in the collagen, and measuring the pH of the solution with the glass electrode. A period of 4 days was allowed for the system to come to equilibrium. The pH of the solution at the end of this time was 7.7. This result is consistent with the fact that collagen contains more basic groups than acidic groups. Also, it affords a simple explanation of the pronounced end point in the titration curve of collagen [3, 4] between the pH values of 7 and 10. As electrophoretic measurements give a value of about 5 for the isoelectric point [2, 10], it may be concluded that the assumption in question is not valid for collagen, and thus can not be valid in general.

III. PREPARATION OF COLLAGEN

The raw materials from which the samples of collagen were prepared were obtained, through the courtesy of the U. S. Department of Agriculture, from a steer slaughtered at the Experimental Station at Beltsville, Md. The bend portion of the hide, the carpal and tarsal flexor tendons, and the metacarpal and metatarsal bones were used.

With certain exceptions, as noted below, the materials were processed according to the method given by Highberger [5]. The tendons were prepared for the treatment with trypsin by cutting away all muscular tissue and removing as much as possible of the areolar connective tissue from the outside. They were then cut into sections about 1 cm in length. The bones required more drastic preliminary treatment. The ends were cut off, the marrow was removed, and the periosteum was scraped off. They were then put into a 0.1 *N* solution of hydrochloric acid, covered with a layer of xylene. The solution was changed every day or two for a month, and after this twice a week for three more months. At the end of this time the material was cut into small pieces, and further washed with dilute hydrochloric acid until no opaque regions remained. The effect of

this long treatment with acid appeared when the amount of the material dissolving in alkaline solutions was determined. A much larger quantity of nitrogen was found in solution with the bone collagen than with either the hide or tendon. Hydrochloric acid was used for the final removal of calcium from all three materials.

The purified materials were analyzed for chlorine and calcium. The chlorine contents of the samples, expressed as the ratio of the number of gram-atoms of chlorine to the number of gram-atoms of nitrogen in the sample, were 0.0048, 0.0005, and 0.0044 for the hide, tendon, and bone, respectively. The calcium contents of all the samples were less than 0.0002, expressed in the same way. A qualitative test for phosphate in the bone collagen gave a negative result. The ash content of the hide collagen (determined after adding sulfuric acid) was 0.05 percent.

IV. EXPERIMENTAL PROCEDURE

The samples of collagen were conditioned and weighed in an atmosphere at 65-percent relative humidity and 22° C 0.97 g of collagen, 20 ml of hydrochloric acid or sodium hydroxide solution of the desired concentration, and an excess of solid sodium chloride were put in a glass-stoppered flask. The flask was put in a constant-temperature room at 25° C and allowed to remain, with occasional shaking, for 25 hours. Part of the solution was then poured into a test tube, the tube was stoppered, and the suspended solid matter was allowed to settle. After settling, a 10-ml sample of solution was pipetted into a flask for titration. The titration was carried out volumetrically with 0.05 N sodium hydroxide or hydrochloric acid, using methyl red as the indicator. The titration was run past the exact end point of the indicator, as it was necessary, for the reason given above, to neutralize all the acid or base in the solution, without regard to its combination with dissolved matter from the collagen. Finally, the total nitrogen in the solutions titrated was determined.

V. CALCULATION OF RESULTS

The mass of water in a unit volume of a saturated aqueous solution of sodium chloride containing a small amount of hydrochloric acid or sodium hydroxide is practically independent of the concentration of acid or base [6, 7, 8, 9], and is equal to 0.882 g/ml at 25° C. In this work the effect of the dissolved nitrogenous matter on the concentration of water was neglected.

The total mass of water in the system was taken as 0.03 g less than the mass of water added. This difference represents the amount of water taken up by the collagen in passing from 65- to 75-percent relative humidity. This correction was made because the collagen used in the work cited above [1] was conditioned at the higher humidity.

The fundamental equation used for calculating the results was $A=f\left(N-H\frac{n}{h}\right)+H\frac{a}{h}$, where A is the total acid or base in the system (including that introduced with the collagen), N is the total nitrogen, H is the total mass of water, and f is the desired ratio of acid or base fixed to undissolved nitrogen. The lower-case letters refer to the amounts of the same components in the part of the system taken for

titration. A and N are expressed in equivalents. This equation expresses the fact that the total acid or base is the sum of that part fixed by the undissolved collagen, and the part in solution. The ratio f is thus given in the symmetrical form

$$f=\frac{A-H\frac{a}{h}}{N-H\frac{n}{h}}.$$

The equilibrium concentration was calculated under the assumption that the ratio of combined acid or base to nitrogen was the same for the dissolved and undissolved portions. There is no assurance that this is a good approximation, but the concordance of the results indicates that the concentration calculated in this way is satisfactory for use as an independent variable in presenting the results. The molal concentration of acid or base was calculated from the expression $m=(A-fN)/H$.

VI. DISCUSSION OF RESULTS

The experimental results are given in tables 1 and 2, and in figure 1.

TABLE 1.—*Combination of collagen with hydrochloric acid*

[m is the molal concentration of acid in the equilibrium solution. f is the ratio of the number of equivalents of acid combined to the number of equivalents of nitrogen in the sample.]

Hide		Tendon		Bone	
m	f	m	f	m	f
0.002	0.070	0.004	0.074	0.005	0.072
.006	.071	.017	.077	.018	.074
.011	.073	.039	.080	.039	.079
.016	.074	.064	.085	.066	.082
.022	.075	.098	.092	.100	.088
.030	.076				
.039	.079				
.051	.080				
.066	.083				
.088	.088				

TABLE 2.—*Combination of collagen with sodium hydroxide*

[m is the molal concentration of base in the equilibrium solution. f is the ratio of the number of equivalents of base combined to the number of equivalents of nitrogen in the sample.]

Hide		Tendon		Bone	
m	f	m	f	m	f
0.002	0.010	0.005	0.022	0.003	0.025
.003	.016	.017	.028	.014	.034
.006	.020	.038	.030	.034	.039
.010	.023	.064	.032	.060	.041
.015	.025	.098	.033	.091	.047
.022	.027				
.031	.027				
.042	.029				
.056	.029				
.074	.030				

Perhaps the most noticeable thing in figure 1 is the difference between the results for the bone collagen and those for the other two materials in the alkaline solutions. It is natural to look for the source of this difference in the treatment used for deliming the bones. There is no reason to suppose that peptide linkages were hydrolyzed by the long treatment with acid, because the amount of acid combined is the same for the bone as for the hide and tendon. If unsubstituted amide groups were hydrolyzed, however, the amount of base bound would increase without any change in the combining weight with respect to acid. A comparison of the amounts of ammonia nitrogen

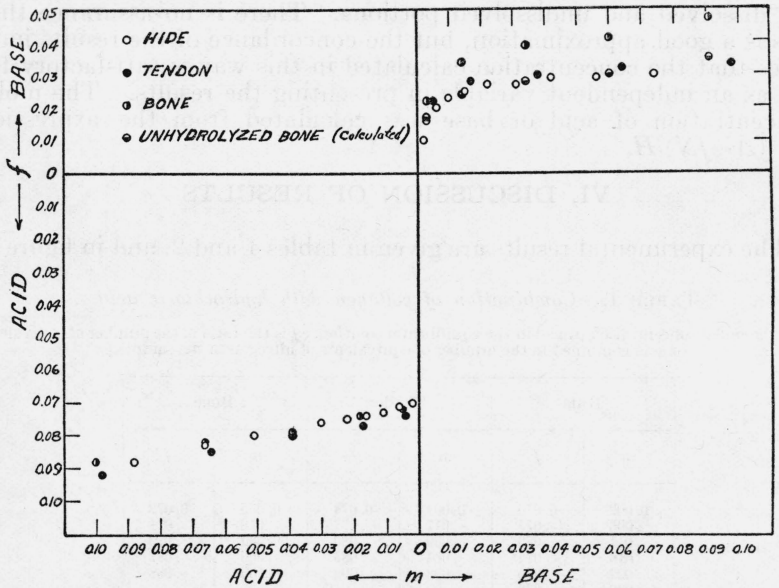

FIGURE 1.—*The combination of collagen with acid and base.*

The abscissas represent the molal concentrations of hydrochloric acid or sodium hydroxide. The ordinates represent the amounts of acid or base combined, expressed as the ratio of the number of equivalents combined to the number of equivalents of nitrogen in the sample.

in acid hydrolysates of the hide and bone collagens showed that the fraction of the total nitrogen as amide nitrogen was less by 0.010 in the bone collagen.

It is possible to calculate approximately what the amount of base combined with the bone collagen would have been if carboxyl groups equivalent to this amount of nitrogen were replaced by amide groups, assuming that the fraction of the number of carboxyl groups present which react with the base is a function only of the concentration of base. It appears from figure 1 that the carboxyl groups in the collagen are almost completely neutralized even at low concentrations of base, and that additional base is taken up by some noncarboxylic groups. The amount of this additional base combined seems to vary

nearly linearly with the concentration, in the range of concentrations covered. The calculation in question was made on the basis of these observations. The constants in the linear relation between f and m for the bone collagen were evaluated by the method of least squares, using the three highest values of m and neglecting the errors in m. If the four highest values of m are used the results are practically the same. The only object in using the method of least squares here was to make the calculation as nearly objective as possible. The constant term in the equation giving f as a function of m ($f=0.033+0.15\,m$) was taken to represent the limiting amount of base combining with carboxyl groups, the first degree term to represent the additional amount of base combined. Then, if 0.010 (the difference in the fraction of the total nitrogen as amide in the bone and hide collagens) is subtracted from the constant term, the difference is the calculated value of the limiting amount of base combined with carboxyl groups, for the modified bone collagen. The ratio of this difference to the constant term, $(0.033-0.010)/0.033$, gives the factor for converting the amount of base combined with carboxyl groups in the bone collagen to the amount for the modified bone collagen. Then the calculated f is given by the expression

$$\frac{0.023}{0.033}(f-0.15\,m)+0.15\,m.$$

Figure 1 shows the comparison of these calculated values with the observed values for the hide and tendon. It may be seen that the difference between the amide nitrogen contents accounts for the difference between the amounts of base combined. It seems proper, therefore, to conclude that the white fibrous connective tissues of hide, tendon, and bone behave alike with respect to combination with acid and base, and that the drastic treatment with acid hydrolyzed a considerable fraction of the amide groups in the bone collagen.

VII. SUMMARY

Collagen from three different sources, hide, tendon, and bone, was purified by the method given by Highberger [5]. The amounts of hydrochloric acid and sodium hydroxide taken up by these materials from dilute solutions of acid or base, saturated with sodium chloride, were measured. Four of the possible sources of error in the measurement are discussed, and the methods used for overcoming them, or the assumptions involved in disregarding them, are outlined. The pH of a solution in equilibrium with neutral hide collagen is found to be in the neighborhood of 8. The three samples of collagen are found to combine with equal amounts of acid, but the bone collagen combines with more base than do the other two. This difference is accounted for by a loss of amide nitrogen during the preliminary treatment of the bone collagen. It is concluded that the white fibrous connective tissues from the three sources exhibit the same behavior toward acids or bases.

VIII. REFERENCES

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