

DETERMINATION OF THE ISOIONIC POINT OF COLLAGEN BY THE BUFFER SHIFT METHOD*

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ABSTRACT

A rapid, yet accurate, determination of the point of zero net charge was required for the characterization of samples of collagen. Samples were equilibrated with dilute buffer solutions and the change in pH of the buffer measured. The buffer shifts gave smooth curves when plotted; the point of zero shift was taken as the isoionic point. Certain buffers, notably phthalate, gave clear evidence of interaction with the protein, for the isoionic point was shifted markedly from that given by other buffer systems. This method requires the use of a sample free of combined acid or base. There do not appear to be any other limitations that are not also pertinent in electrophoretic measurements of the isoelectric point.

INTRODUCTION

In studies of the purification of collagen from hide, a simple and rapid method of determining the isoionic point of various preparations was needed. Purified steerhide collagen has an isoelectric point in the region of pH 7-8, as determined by acid-base titration (1, 2). As it occurs in hide, it is combined with an acid mucoprotein, the combined product, or crude collagen, thus having a lower isoelectric point. Furthermore, the liming of hides in tanning results in an alkaline hydrolysis of the carboxy amide side chain groups of glutamine and asparagine which occur in collagen. The free carboxy groups which appear contribute negative charges, with a resultant fall in the isoionic point to a pH of about 5.0. Thus a determination of the isoionic point of samples of known history gives information as to the success of extraction of impurities and of the amount of breakdown of collagen during processing.

The moving boundary method of Tiselius (3) has become the preferred method of determining the isoelectric point of soluble proteins, but it is not applicable

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to insoluble proteins. The determination of electrophoretic migration of microscopic particles in the Northrup-Kunitz method (4), which can be used, is tedious and not well adapted to routine practice. Recourse was therefore made to the determination of the shift in pH of dilute buffer solutions.

The point of zero shift, when the change in pH of the buffer is plotted against the final pH, is a sensitive and relatively accurate measure of the isoionic point. This procedure, originally employed by Michaelis (5) to determine the isoionic point of the amino acids, glycine and phenylalanine, was later used by Miyake to characterize a number of prolamines in the region of pH 12 (6). Hitchcock (7) compared the available methods of determining the isoelectric and the isoionic points of proteins in a careful study of gelatin in 1931.

A full discussion of the terms, isoelectric point and isoionic point, does not come within the limits of this presentation, but certain definitions are essential. The isoelectric point is the pH of zero migration in an electric field under specified conditions of ionic strength and buffer composition. The isoionic point is defined as the pH at which the number of protons combined on the basic groups is equal to the number dissociated from the acidic groups. Therefore, at the isoionic point the average net electric charge is zero. For the present purposes, the isoionic point is that pH at which the added protein does not change the pH of a solution. This point is a function of the ionic strength of the solution and may vary with different buffer systems if interaction between the buffer and the protein occurs. Thus, the conditions of temperature, ionic strength, composition of the buffer, and concentration of suspended protein must be stated. The buffer shift method suffers from the disadvantage that the sample must be free of combined foreign ions. Electrophoretic migration in buffers, on the other hand, is not affected by the presence of small amounts of foreign ions if the latter are readily dissociated from the protein.

EXPERIMENTAL

In our work collagen was prepared from steerhide corium by a combination of steps which included extraction with NaCl, acetone, ether, half-saturated lime water, dilute acetic acid, and water. The final product was washed exhaustively and air-dried. It was ground in a Wiley mill to pass through a 1/32-inch screen. The final product contained 17.93 percent nitrogen and 0.17 percent ash on a moisture-free basis. The moisture content was 15.35 percent. The hide powder was the American Standard product, lot number 25, ground in a small Wiley Mill through a 20-mesh screen. It contains 17.87 percent nitrogen, 0.18 percent ash (moisture-free basis), and 12.5 percent moisture.

In the actual experiments, one-gram samples (air-dried) of protein were placed in one-ounce vials, and 20 ml. of the respective buffer was added. The suspension was turned end-over-end in a water bath at $25.0 \pm 0.10^{\circ}\text{C}$. for two to six hours. Tests showed that the ground samples were equilibrated at the end of two hours.

The pH of the sample was determined by a Beckman model G pH-meter.** The electrodes could be inserted in the test vials directly, without removal of the sample mixture. The pH-meter was calibrated with standard buffers and the calibration is believed to be accurate to ± 0.02 pH unit. The buffer shift is certainly valid to this same limit, for it is a differential measurement.

The acid-base titration curve was measured at approximately constant ionic strength of 0.05, using NaCl with appropriate correction for the blank titration. These results were not of high accuracy but were sufficient to determine the nature of the curve in the isoelectric region for comparison with the values obtained by the buffer shift method.

RESULTS

The acid-base titration curves shown in Figure 1 are similar to those previously obtained for collagen (8) and hide powder (9). The major features of the curves for this discussion are the small slope of the collagen curve in the region of the

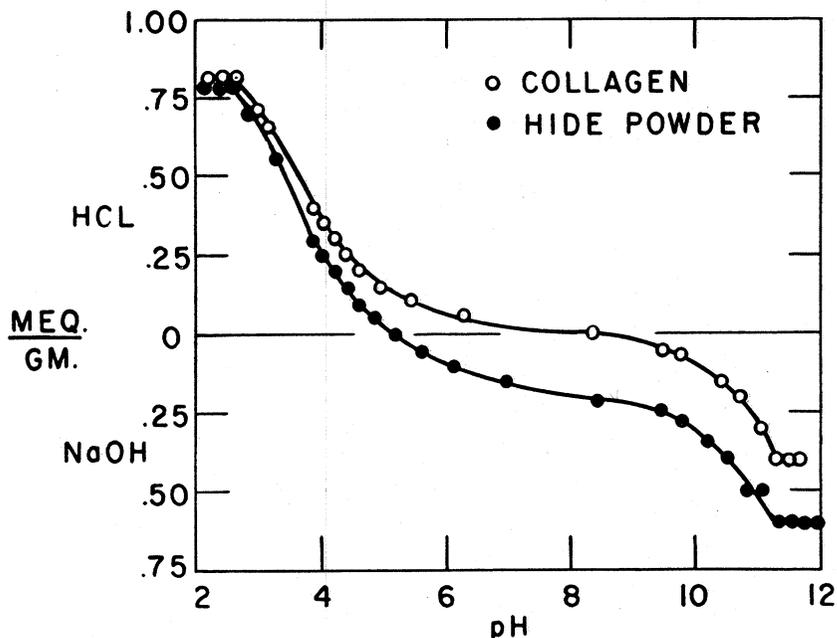


FIGURE 1.—Acid-base titration of collagen and hide powder.

isoelectric point at pH 8.3, which means that there are few dissociable groups in this region and a small buffer capacity. Hide powder, on the other hand, has a marked buffer action in its isoelectric region at pH 5.2 and consequently buffer

**Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

shift determinations can be made at higher buffer concentrations. The marked similarity of the two curves in the alkaline portion is noteworthy; the two curves are almost superimposable. The major difference between the two curves lies in the acid portion, where the increase in carboxy groups, which titrate between pH 2.5 and 6.0, is apparent in hide powder.

A series of determinations of the isoionic point of hide powder with different concentrations of citrate buffer is shown in Figure 2. The isoionic points are pH 5.19, 5.15, and 5.14 for 0.100, 0.025, and 0.005 molar buffer, respectively. There

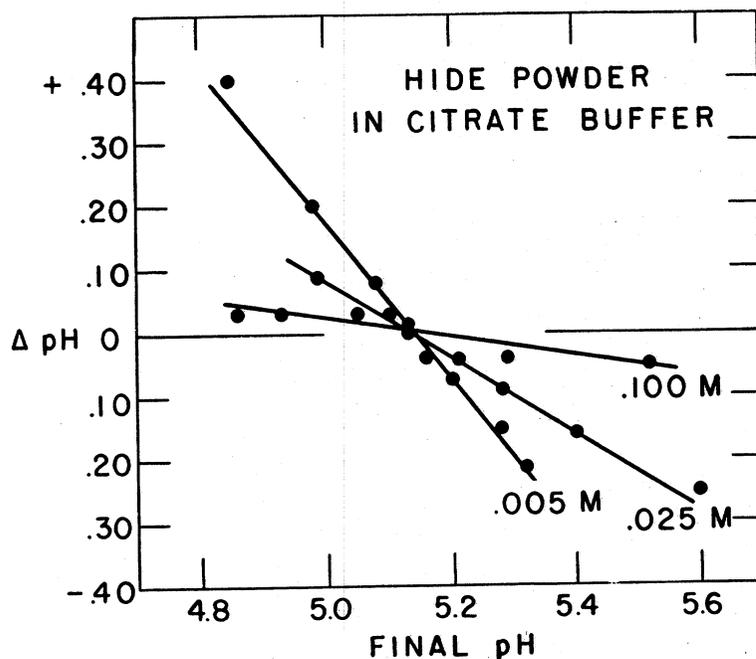


FIGURE 2.—Isoionic point of hide powder in citrate buffer.

is only a small effect of buffer concentration in this series, but the values obtained with 0.001 molar buffer was definitely lower at pH 4.94. Similar determinations were made with phthalate and acetate buffers in an effort to observe specific effects due to the buffer ions. The results in Table I show that the effect of buffer concentration does differ with the different buffers. The phthalate buffer has a large concentration effect, whereas the other two buffers give comparable results in the same molar concentrations.

Thousandth molar buffers were made up in 0.2 molar NaCl and both the phthalate and the citrate buffer systems showed a marked increase in the isoionic point of hide powder on raising the ionic strength. It has been reported by many workers that the ionic strength of the medium has a marked effect on the iso-

TABLE I
ISOIONIC POINT OF HIDE POWDER

Buffer System	Molarity			
	0.001	0.005	0.025	0.100
Phthalate	4.96	4.98	5.28	5.38
Citrate	4.94	5.14	5.15	5.19
Acetate	—	5.10	5.00	5.20

electric point of many protein-buffer systems, notably gelatin buffer systems. Hitchcock (7), however, reported that the isoionic point of gelatin is independent of the ionic strength. This is rather surprising in view of the fact that gelatin is produced from collagen by very mild treatment.

Similar determinations of the isoionic point of collagen were made. The results are shown in Table II. The data at buffer concentrations above 0.100 molar were not satisfactory; the shifts in pH were too small. Veronal and borate buffer gave comparable results. The effect of ionic strength is especially noteworthy in this poorly buffered system; the isoionic point is raised a full pH unit by the

TABLE II
ISOIONIC POINT OF COLLAGEN

Buffer System	Molarity			
	0.001	0.005	0.010	0.001 in 0.200 M NaCl
Veronal	7.62	8.00	8.20	8.63
Borate	7.72	7.97	8.18	8.76

addition of 0.200 molar NaCl. For convenience and accuracy of measurement, the preferred buffer solutions appear to be 0.001 molar in 0.20 molar NaCl. The pH measurements on the buffers are satisfactory, although the pH of the buffer solutions at this low molarity differs markedly from that of the standard Sorenson 0.10 molar buffer. The pH shifts are large, and the isoionic point can readily be estimated within 0.020 pH unit by inspection of the data.

Several of the isoionic points were checked on samples of protein which had been electrodyalized to remove the small amount of ash. The pH values were not different, but it must be remembered that this method will give false results if combined acid or base is present in the sample.

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DISCUSSION

DR. TU: Dr. Levy will lead the discussion.

DR. LEVY (Rohm and Haas Company): Thank you for a very fine paper, Al.

The method that Mr. Korn has outlined for determining the isoionic point of collagen and of hide powder is noteworthy in that it requires relatively inexpensive instrumentation. It's one of the few times when accurate, precise, and significant research on the collagen molecule can be done with equipment costing less than several thousands of dollars.

Since the processing of hides, the tanning and the liming, does affect the chemical structure of the collagen molecule, and does result in hydrolysis of certain side groups, the isoionic point of collagen is changed. Treatment of collagen with various reactive chemicals probably will also change the isoionic point, and therefore having a fast and simple method available to determine these changes in the collagen molecule will, I think, aid all of us who are actively engaged in the study of collagen chemistry.

I would like to ask Mr. Korn whether or not this method is really just limited to the use of insoluble materials for determining isoionic points, or whether it can be used for soluble collagen as well.

MR. KORN: Although we haven't run any soluble proteins using our method, these systems could be expected to cause a shift in the pH of dilute buffer solutions. However, it would be more desirable to use the Tiselius method for most soluble proteins.

There has been a great deal of discussion about so-called soluble collagen and it was thought that this material could be run by the Tiselius method. Unfortunately, in this method, the sample must be run at about 4°C., where the temperature-density curve for water is nearly flat, so that the convection currents which would destroy the free boundaries are avoided. Under these conditions, soluble collagen would probably reaggregate and might even jell, so that it would be impossible to get any sort of electrophoretic pattern.

DR. LEVY: I'd like to open the paper for questions and discussion from the floor. Since there are no questions, I'd like to thank Mr. Korn for coming here today to share his research with us.