In a study of the acid- and base-binding capacity of fibrin and fibrinogen, it became evident that the latter is changed by long standing in the presence of a small amount of acid, even when the solution is kept in the refrigerator. The change is shown by the greater tendency of the protein to precipitate. Unless the protein has been previously dialyzed against sodium chloride solution, it forms an insoluble jelly-like precipitate soon after the addition of enough hydrochloric acid to bring the pH to 2.5 or 3.0. This precipitate usually does not form if the protein has been dialyzed. That the dialyzed fibrinogen is also changed by acid is shown by the appearance of a precipitate when the acid is neutralized. This precipitate does not dissolve readily in dilute alkali, whereas native fibrinogen is easily dissolved by a small amount of sodium hydroxide. The fibrinogen is so readily changed by the addition of acid that 0.1 N HCl usually precipitates some of the protein immediately in an insoluble form. A more dilute acid (0.02 N) in equivalent amount may not cause immediate precipitation, but if the fibrinogen solution has not been previously dialyzed, the precipitate is formed after standing several hours in the cold.

The process of heat denaturation of protein has been explained by Robertson (1), by Sørensen (2), by Chick and Martin (3), and others as a dehydration. This dehydration, according to Robertson, is analogous to the loss of water of crystallization by certain salts. Wu and Wu (4) thought that they had evidence to show that hydrolysis of certain peptide linkages occurred during the process of coagulation. More recently, Wu (5) has concluded that such hydrolysis is not an essential part of coagulation. P. S. Lewis (6) suggested that hydrolysis occurs during coagulation of proteins but that this hydrolysis does not involve the peptide linkage. W. C. M.
Lewis (7) had previously advanced the idea that coagulation was brought about by the opening of some bond in the protein molecule other than the --CO—NH— bond but definite evidence of the nature of such a bond is not given. Booth (8) has titrated coagulated and uncoagulated egg albumin in the presence of ammonium sulfate and found no reason to believe that the acid- and base-combining power of that protein is changed during the process of heat coagulation. Hendrix and Wilson (9) found that coagulated egg albumin combines with less acid or base than the uncoagulated protein. Booth coagulated his protein at pH 6.99 and on a boiling water bath where the temperature probably did not reach 100°. Hendrix and Wilson heated their material at the isoelectric point in an autoclave at atmospheric pressure for 30 minutes.

Denaturation of protein by acid has not received the same attention as heat denaturation. Wu and Wu (10) investigated the effect of acids and alkalies upon several proteins and found that, in general, the solubility of the protein was altered. Cubin (11) has studied the denaturation of oxy-hemoglobin and of egg albumin at 18–37°, at various hydrogen ion concentrations. Hemoglobin is rapidly denatured at pH 4 even at 18°, while egg albumin is only slowly denatured at pH 1 unless the temperature is about 37°. Cubin gives no evidence to show whether the acid denaturation of protein is similar in effect to heat denaturation.

The results presented in this paper are of interest in that they show that denaturation of a protein by dilute acid produces an effect upon the acid- and base-combining power similar to that of heat denaturation as carried out by Hendrix and Wilson.

Methods

Preparation of Fibrinogen—Beef blood was collected over potassium oxalate at the slaughter-house, brought to the laboratory, and stored in the ice chest. This blood was centrifuged as rapidly as possible but at least 2 days were necessary to centrifuge the entire sample. The usual difficulties in obtaining a clear plasma from beef blood were encountered, thus making necessary several precipitations of the fibrinogen. The precipitations were carried out with sodium sulfate, the final concentration being about 10 per cent, as suggested by Howe (12). 300 gm. of sodium sulfate were dissolved with the aid of heat in water, filtered, the volume adjusted to 750 cc., and the solution at a temperature of 40° poured into 3 volumes of the cold plasma. The copious precipitate was separated by centrifuging, washed several times with half saturated sodium chloride solution, and reprecipitated. Only
rarely were two precipitations sufficient, usually three being necessary to remove the hemoglobin. The final precipitate was washed well in half saturated sodium chloride, dissolved in the dilute saline, filtered, and analyzed for nitrogen (Kjeldahl) and for chloride (Volhard-Harvey). Sometimes difficulty in redissolving the protein was encountered after the third precipitation. A few cc. of 1 per cent sodium bicarbonate served to dissolve the fibrinogen in such cases. Dialysis was made part of the routine procedure in the later part of these experiments, in order to make sure that no sulfate remained in the solution, although repeated analyses had shown none to be present. Before filtration, the protein was rapidly dialyzed against running sodium chloride solution, about 18 liters being used. It was then placed in the ice chest and allowed to dialyze overnight against large amounts of the saline. The next morning the protein was again dialyzed against 18 liters of running saline, filtered, and the analyses made. From the nitrogen analysis the protein content of the solution was calculated with the factor 6.25 and volumes containing 0.5 gm. of fibrinogen were measured out.

This procedure usually took 4 days, 2 for centrifuging, 1 for precipitation and purification, and 1 for analysis. Throughout this time the protein was kept at a low temperature and from the physical properties and the speed of clotting when thrombin was added no denaturation seemed to take place. The protein stayed in solution very well unless through error the sodium chloride content was too low or the protein content too high. The yield varied from 6 to 22 gm. from 12 liters of blood.

Titration—The 0.5 gm. portions of protein were diluted and the sodium chloride content adjusted. The volume, no matter what the treatment, was always brought to 125 cc. and the sodium chloride was made the same for all members of a series. At first 0.9 per cent sodium chloride was used but later 1.25 per cent was found more satisfactory. After dilution one series was allowed to stand overnight in the ice chest without further treatment. To the remainder were added 35 cc. of 0.02 N HCl, the final concentration of acid thus being about 0.0056 N. This gave a pH which in various experiments ranged from 2.5 to 3.5. The acid-treated series was also allowed to stand overnight with the first series.
Acid Denaturation of Fibrinogen

On the following day, varying amounts of 0.02 \( \text{N} \) \( \text{NaOH} \) were added to the acid-treated samples to give a series ranging from the original pH (2.5 to 3.5) to about pH 8. After mixing, the pH was determined immediately with either the hydrogen or quinhydrone electrode. We found that the two electrodes checked each other fairly well within the range used in this work. In experiments upon the undialyzed material, a precipitate always formed on standing with the acid, which made thorough mixing with the alkali difficult. With the dialyzed material, a heavy precipitate always formed near the isoelectric point, which again gave mechanical difficulties; but the agreement between the dialyzed and undialyzed material seems to show that both were mixed thoroughly.

Two methods were employed with the untreated material. At first, the volume was adjusted to 90 cc. to stand overnight and just before titration 35 cc. of the 0.02 \( \text{N} \) \( \text{HCl} \) were added, plus the appropriate amount of \( \text{NaOH} \). Later on we found that identical results were obtained if instead of adding acid plus base, the desired amount of acid or alkali was added directly to the protein. The volume difference was adjusted by means of a control. The method of Tague (13) was used, control determinations of sodium chloride solution being made and the amount of acid or alkali necessary for the control subtracted from the protein figure. Thus the curves (Charts I and II) represent volumes of acid or base combined with 0.5 gm. of protein, with the isoelectric point\(^1\) taken as zero.

\(^1\) The isoelectric points of the acid-treated and of the untreated fibrinogen were determined by a modification of the method of Cole (14). Measured amounts of the protein solution were added to mixtures of acetic acid and sodium acetate and the pH of the tube containing the greatest precipitate determined by the quinhydrone electrode. Some difficulty in judging the amount of precipitate was encountered with the acid-treated protein, as there was a heavy precipitate in all tubes near the isoelectric point. However, the results in a number of determinations agreed quite well and the average of these various experiments was taken for the zero point on the curves. In the acid-treated protein, the isoelectric point shifted towards neutrality as would be expected. The values obtained were 4.686 for the untreated and 5.20 for the acid-treated. The determinations were made on almost all of the untreated preparations and on all the acid-treated samples which did not precipitate.
In some instances the untreated protein and the acid-treated protein were analyzed for amino nitrogen by Van Slyke's manometric method.

DISCUSSION

In Chart I are plotted the results of eleven titration experiments upon untreated fibrinogen. Chart II shows the titrations upon the corresponding acid-treated samples. The probable curves have been drawn in both charts. The observed variations are probably caused by varying amounts of impurities in the fibrinogen samples. The original pH of the solutions before treatment ranged from 5.67 to 6.99. The mechanical difficulties in preparing a pure fibrinogen solution are great since its chief difference from other globulins is the reaction with thrombin. In testing a solution with thrombin, globulins may be carried down with the coagulum. In certain samples the serum from a stiff clot would repeatedly show further coagulation upon additions of thrombin, thus making difficult a quantitative determination by the clotting method.

The difference in the curves in Charts I and II shows that the acid- and base-combining power of the acid-treated fibrinogen is less than that of the untreated fibrinogen. The extent of this decrease is shown readily by the use of Van Slyke's formula (15) for buffer value. When $\beta$ is the buffer value, $\Delta B$ is the increase or decrease in base and $\Delta \text{pH}$ is the change in pH. 

$$\frac{\Delta B}{\Delta \text{pH}} = \beta$$

With an increase or decrease in base equivalent to 1 cc. of 0.02 N base as the unit for $\Delta B$ and a shift of 1 pH as the unit for $\Delta \text{pH}$, $\beta$ has been calculated for the untreated and for the acid-treated protein between pH 3 and 8.5. The results are given in graphic form in Chart III. The buffer value for untreated fibrinogen is higher throughout the whole range of our titrations. This shows distinctly that more base is necessary to change the pH 1 unit in an untreated fibrinogen solution than in an acid-treated solution. Since $\beta$ is the slope of the curve the results are not affected by any error in the determination of the isoelectric point of the protein.

The difference in the buffer value of the acid-treated and the untreated fibrinogen appears to be greatest in the region of pH 3 and least from pH 8 to 9. These are the regions where the com-
Chart I. Composite of eleven curves, showing the volume of 0.02 N HCl or NaOH combined with 0.5 gm. of untreated fibrinogen, with isoelectric point at pH 4.686.
CHART II. Composite of eleven curves, showing the volume of 0.02 N HCl or NaOH combined with 0.5 gm. of acid-treated fibrinogen, with isoelectric point at pH 5.20.
The composite curve is least accurate. In the high acid range, the slope of the curve towards zero found in the individual combination curves tends to be lost in the composite curve. On the alkaline side, the titration enters the region where the quinhydrone electrode is less accurate. Therefore B can be most accurately compared in the region from pH 4 to 7.5. At pH 4 the buffer value of the untreated protein is greater than that of the acid-treated by 2.86 and at pH 6.8 the difference is 1.2, showing some tendency for the values to approach each other on the alkaline side. At pH 3 the difference is nearly 7, while at pH 8.2 it is about 0.6.

The difference in acid- and base-combining power of native and of acid-denatured fibrinogen is similar to the difference in the combining power of untreated and heat-coagulated egg albumin, observed by Hendrix and Wilson. It is impossible to compare accurately the observed effect of acid treatment with that produced by heat coagulation because different proteins have been used and because the amount of the two taken for titration was...
not the same. We can conclude that fibrinogen denatured by
dilute acid at a low temperature shows a decrease in acid- and
base-combining power similar to that observed for egg albumin
coaagulated at the isoelectric point by autoclaving at atmospheric
pressure for 30 minutes. These results are in accord with the
theory that during denaturation protein undergoes polymeriza-
tion. Some confirmatory evidence for this point has been ob-
served by determining the primary amino nitrogen of the untreated
and the acid-treated fibrinogen. These determinations have not
been completed but have shown about 8 per cent less amino nitro-
gen in the acid-treated than in the untreated fibrinogen.

SUMMARY

Fibrinogen from beef blood is denatured by standing with hy-
drochloric acid at pH 2.5 to 3.5 for 12 to 15 hours at 12–14°. The
acid- and base-combining power of fibrinogen is diminished by
this acid denaturation. The acid-denatured fibrinogen has an
isoelectric point of pH 5.2, the undenatured, one of 4.7. The
buffer values of the untreated and of the acid-treated protein
have been calculated from the combining curves. The buffer
value of the acid-treated fibrinogen is less than that of the un-
treated protein throughout the entire range of our titrations.

BIBLIOGRAPHY

1. Robertson, T. B., Principles of biochemistry, New York and Philadel-
14. Cole, S. W., Practical physiological chemistry, Cambridge and Balti-
more, 7th edition, 78 (1926).
THE EFFECT OF ACID DENATURATION UPON THE COMBINING POWER OF FIBRINOGEN
Marion Fay and Byron M. Hendrix


Access the most updated version of this article at http://www.jbc.org/content/93/2/667.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/93/2/667.citation.full.html#ref-list-1