STUDIES ON THE EFFECT OF ALKALI ON PROTEIN

I. THE OPTICAL BEHAVIOR OF "RACEMIC PROTEIN"

BY FRANK A. CSONKA AND MILLARD J. HORN

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington)

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Preliminary to a study of the optical rotation of glutelins (1), we carried out experiments on the effect of the concentration of alkali at different temperatures on protein. The chemical changes occurring in the alkali digest were followed optically and also by determining the nitrogen not precipitable with tungstic acid. The change in optical rotation is practically parallel with the decrease of protein nitrogen precipitated, pointing to a rapid disintegration of the protein molecule. These results led us to separate and study the protein cleavage products, which were called by Dakin "racemized proteins."

The polariscopic readings of these alkali digests never reached zero activity. We believed that this optical activity could be explained by the presence of optically active substances in the filtrate. Several papers have appeared in the past, by Kossel and Weiss (2), Dakin (3), and Underhill and Hendrix (4), on the effect of alkali on proteins and the chemical and physiological behavior of the products obtained. All these authors must have considered the products optically inactive or closely so, but to our knowledge their specific rotation was never determined. One may obtain from the literature a misleading conception in regard to the nature of these so called racemized proteins. For instance, the expression racemic casein naturally conveys the idea of an optically inactive protein. As a matter of fact, so called racemic casein is not casein but a decomposition product of casein, and it is optically active. This would be true even if casein is a mixture of several proteins, according to the present belief. It would
be more satisfactory at present to consider these products as opti-

cally active decomposition products of proteins and drop the word
"racemized" entirely. We are not ready to suggest a specific
name for them, but on account of their characteristic behavior
toward acid hydrolysis as contrasted with that of native proteins
they should be in a class by themselves. When hydrolyzed by
acid the native proteins yield optically active amino acids, and a
very small quantity of racemized amino acids. On the other hand,
Dakin showed that when the protein products obtained by alkali
treatment are hydrolyzed by acid, they yield racemic amino acids
and only a small quantity of active amino acids.

We prefer to make a distinction between the effect of alkali on
proteins and the result of acid hydrolysis of the cleavage products
separated from the alkali digest. Racemization occurs in the
latter procedure; the alkali produces only some unknown change in
the molecule of the protein cleavage products, for which we can
offer only a plausible theory, which, however, we believe is more
in harmony with our experimental findings than is Dakin's. That
the optical rotation of the alkali digest is less than that of the
native protein could be explained satisfactorily without consider-
ing the phenomenon of racemization. The optical rotation of
the alkali digest represents the sum of the optical activities of the
cleavage products, which is lower than the activity of the native
protein. The fact that we were unable to separate any racemic
product from the alkali protein digest supports our hypothesis.

Dakin's theory of protein racemization involves the change of
an asymmetric carbon atom into a symmetric carbon atom. How-
ever, the different protein fractions separated from alkali digests
are all optically active. It is true that he found optically active
amino acids in the acid hydrolysate of the so called racemized
proteins, which he attributed to the position of some amino acid
having a free carboxyl group in the protein molecule. It would
be rather far fetched to assume that the optical activity possessed
by these alkali cleavage products is caused by the amino acids
having a free carboxyl group and that after acid hydrolysis the
optical activity is practically lost instead of remaining almost con-
stant. We say almost constant, as we realize that the part of the
protein molecule to which the side chain carboxyl groups are
attached undoubtedly influences the optical rotation in some meas-
ure, even though the attached groups themselves are inactive; the transitory enol formation, according to Dakin, makes them inactive.

Our explanation for the change of optical activity when protein is treated with alkali is that the protein products have a specific rotation of their own, which is different from that of the original protein. All these products obtained from the alkali digests were found to be active. We maintain that in them the asymmetric carbon atoms remain asymmetric, hence their optical activity. There must exist, however, chemically or physically a difference in the molecule of these products and in that of the native proteins, as acid hydrolysis results in optically inactive compounds in the first case and optically active compounds in the second case. This difference is probably caused by the manner in which alkali breaks down the protein molecule or shifts certain groups within it with reference to the asymmetric carbon atoms.

Levene and Bass (5) digested proteins with alkali for different time intervals. The products of these digestions were then hydrolyzed with acid. From the results obtained they concluded that racemization of proteins increased with the time they were subjected to the action of alkali. We have shown that there is a rapid disintegration of the protein molecule on treatment with alkali and interpret their findings as confirmatory evidence of our hypothesis that hydrolysis of the protein by the alkali is also a factor in the racemization phenomenon.

When a protein molecule splits, the number of side chain carboxyl groups necessarily increases if the break occurs at the CO—NH peptide linkage; therefore, if Dakin's explanation that the active amino acids found in the hydrolysate of the alkali digest represent the end carboxyl groups is correct, the splitting must occur at some other points. Considering Levene and Bass' observation mentioned above and ours, however, we may assume with Dakin that enolization of the asymmetric carbon atom occurs only on a cleavage of the protein molecule, rendering the secondary end carboxyl groups inactive. This would leave open the explanation for the presence of inactive amino acids in the hydrolysate of optically active alkali cleavage products. Our hypothesis would explain this phenomenon.
EXPERIMENTAL

Preliminary experiments had convinced us that when treated with dilute sodium hydroxide in concentrations of 0.05 and 0.5 M even at room temperature (21°) native protein disintegrates and that when the temperature is raised to 38° this hydrolytic process increases very rapidly. As shown in Figs. 1 and 2, in which are given the percentages of non-protein nitrogen plotted against time, within 48 hours practically one-half of the original protein nitrogen is converted into a form not precipitable by tungstic acid. It is interesting to compare these curves with the racemization curves we published in a previous communication (1) in which we stated that decrease in optical rotation may have nothing to do with racemization. Cleavage of the protein molecule by the alkali and the decrease in optical rotation represent a similar type of reaction and, as we stated above, racemization does not play any part at this stage.

3 to 5 gm. of the protein under investigation were dissolved in
0.05 or 0.5 m sodium hydroxide solution. The solution was filtered in order to remove undissolved particles, and it was then brought by additional sodium hydroxide solution to such a volume that the protein concentration was approximately 1 per cent. Aliquot parts were removed for total nitrogen determinations at the beginning of the experiment. Later at regular time intervals 10 cc. samples were removed for the non-protein determinations as given below. After neutralization with dilute sulfuric acid, equal volumes of 10 per cent solution of sodium tungstate and 0.6 N sulfuric acid were added to precipitate the proteins as recommended for blood analysis by Folin and Wu (6). After standing for an hour, the precipitate was removed by filtration, and the nitrogen content of the filtrate was determined in duplicate according to Pregl's micro method as modified by Clark and Collip (7).
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In Table I are presented data on the cleavage products separated from alkali digests of proteins. We used wheat gluten, casein, and egg albumin. As the technique varied in each case, a description of each experiment is given. We do not claim that these preparations represent individual proteins. The main purpose was to determine the optical behavior of products which, judging from the literature, we believed to be optically inactive. We were more interested in the nature of the alkali effect on the proteins than in the yields of each preparation. All fractions showed the characteristic biuret color reaction.

**TABLE I**

*Properties of Protein Cleavage Products Obtained by Treating Protein with 0.5 M Sodium Hydroxide at 38°*

<table>
<thead>
<tr>
<th>Protein used and time digested</th>
<th>Preparation No.</th>
<th>N on moisture-free and ash-free basis per cent</th>
<th>Specific rotation In 0.5 M NaOH</th>
<th>Specific rotation After hydrolysis with 20 per cent HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluten digested for 52 days</td>
<td>1</td>
<td>14.26</td>
<td>-67.5 degrees</td>
<td>+8.6 degrees</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.45</td>
<td>-98.2 degrees</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.83</td>
<td>-97.9 degrees</td>
<td>+2.7 degrees</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14.64</td>
<td>-76.9 degrees</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>13.47</td>
<td>-69.2 degrees</td>
<td>0</td>
</tr>
<tr>
<td>Casein digested for 36 days</td>
<td>1</td>
<td>12.40</td>
<td>-45.8 degrees</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.20</td>
<td>-30.9 degrees</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.40</td>
<td>-46.0 degrees</td>
<td>0</td>
</tr>
<tr>
<td>Egg albumin digested for 21 days</td>
<td>1</td>
<td>13.20</td>
<td>-25.8 degrees</td>
<td>+2.9 degrees</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.00</td>
<td>-19.8 degrees</td>
<td>+2.2 degrees</td>
</tr>
</tbody>
</table>

*Gluten*

*Preparation 1*—This was obtained by slight acidification of the alkali digest, which had stood for 52 days at 38° (Filtrate A). The precipitate was redissolved in 0.5 M sodium hydroxide, and the clear solution was diluted with distilled water to bring the alkali concentration to approximately 0.05 M. Solid ammonium sulfate was then added to make the solution 35 per cent saturated. The precipitate thus obtained was separated by centrifugation.
(Filtrate X'), redissolved by the addition of water (sufficient alkali being present), and reprecipitated by slight acidification. The precipitate was washed free from salt by distilled water and was dried in vacuo over sulfuric acid.

Preparation 2—This could be obtained either by saturating Filtrate X by the addition of solid ammonium sulfate, or by acidification. The latter procedure was used. The precipitate dissolved almost completely in 70 per cent ethyl alcohol. The solution was filtered clear and evaporated in vacuo to a sirupy consistency. When treated with ether, the product was converted to a light yellowish powder.

Preparation 3—To Filtrate A alcohol was added until 80 per cent alcohol concentration was reached. The precipitate, which consisted mostly of inorganic salts, was washed with 80 per cent alcohol, the washings being added to the filtrate. The alcohol was removed by distillation in vacuo. After acidifying, the resulting precipitate was washed with 70 per cent alcohol and finally dehydrated by treatment with absolute alcohol, followed by treatment with ether.

Preparation 4—The filtrate from Preparation 3 was freed from sulfuric acid by adding an equivalent amount of barium hydroxide. The liquid was concentrated in vacuo to a small volume, and enough alcohol was added to bring the alcohol concentration up to 86 per cent. After it had stood in a refrigerator for several weeks, a brown sirup separated. The supernatant liquid was decanted, and the sirup was dehydrated in the usual manner with alcohol and ether.

Preparation 5—The supernatant liquid from Preparation 4 was evaporated in vacuo to a sirup and dehydrated with absolute alcohol and ether.

Casein

The protein in approximately 1 per cent concentration was digested in 0.5 M sodium hydroxide for 36 days at 38°, after which it was acidified with dilute sulfuric acid. The precipitate was washed and dehydrated (Preparation 1). The liquid was brought to 70 per cent alcohol concentration, and most of the inorganic salts were removed by filtration. The filtrate was evaporated in vacuo to a small volume, and 6 volumes of absolute alcohol were
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added. On standing, a sirup separated. This was dried in vacuo over concentrated sulfuric acid (Preparation 2). The supernatant liquid which was decanted from the sirup was evaporated to dryness. The residue is Preparation 3.

Egg Albumin

The procedure was similar to that used for casein in Preparations 1 and 2.

SUMMARY

Wheat gluten, casein, and egg albumin were digested with 0.5 M sodium hydroxide at 38°. The products which were separated from the alkali digest were optically active, but when boiled with 20 per cent hydrochloric acid they yielded hydrolysates which were practically optically inactive. It is suggested that the name "racemized protein" for these alkali digestion products be discontinued because it is misleading. Dakin's theory of keto-enol formation is discussed, and the conclusion is reached that it does not give a satisfactory explanation for the observations reported in this paper.

BIBLIOGRAPHY

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