THE DISTRIBUTION OF THE SULFUR IN CASEIN, LACTALBUMIN, EDESTIN, AND PAPAIN*

BY BEATRICE KASSELL AND ERWIN BRAND

(From the Departments of Biological Chemistry and Urology, College of Physicians and Surgeons, Columbia University, and from the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York)

(Received for publication, May 12, 1938)

Methods for the determination of cystine, cysteine, methionine, and sulfate in proteins have been discussed in recent publications (1-3). A system of analysis has been developed in which each of these constituents is determined by at least two independent methods. In this paper, a comparison of the results obtained by the various methods and some experiments on the effect of the conditions of hydrolysis on the cystine determinations are presented. Hydrolysis with HCl gives satisfactory results, but for different proteins the optimum time of hydrolysis varies. Hydrolysis with H₂SO₄ has not proved suitable for cystine determinations, particularly by the Sullivan method. The results with reduced lactalbumin make it obvious that the Sullivan method cannot be used for protein hydrolysates which contain a mixture of cystine and cysteine.

The sulfur distribution of a number of proteins is reported. With casein, lactalbumin, and reduced lactalbumin it is possible to account for all of the sulfur, while with edestin and a preparation of papain considerable amounts remain undetermined.

Hydrolysis and Methods—For the cystine determinations, the proteins were digested in an inert atmosphere (CO₂ or N₂) with 6 N HCl, 6 N H₂SO₄, or 42 per cent formic acid in 20 per cent HCl

* This report is from a dissertation submitted by Beatrice Kassell in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

435
Distribution of Sulfur

(4) at 130° for varying lengths of time.\(^1\) HI digestion for the Baernstein determinations was carried out as described previously (3).

The HCl and H\(_2\)SO\(_4\) digests were filtered from the humin and analyzed as soon as the hydrolysis was completed, since it was noted that losses of cysteine may occur if the hydrolysate is allowed to stand.

The determinations by the photometric method (cystine and cysteine separately), by the Baernstein method (methionine as volatile iodide and as homocysteine, cystine plus cysteine, sulfate as H\(_2\)S), and the gravimetric determination of sulfate were carried out as described previously (1, 3).

For the Sullivan method, the procedure described by Brand, Harris, and Biloon (5) was followed, but 10 seconds\(^2\) were allowed between the addition of naphthoquinone sulfonate and sulfite. Suitable aliquots of the hydrolysates were neutralized to pH 7 shortly before the determination. The recent modification recommended by Sullivan and Hess (8) for the determination of cystine in urine was tried, but the results indicated that it is not advisable to use this modification with protein hydrolysates. In proteins containing cysteine, the “cystine” determination by the Sullivan method is too high, since cysteine develops about 1.5 times (9, 10) as much color as cystine (cf. “Reduced lactalbumin”).

In a number of instances, HCl or H\(_2\)SO\(_4\) hydrolysates were treated with cuprous chloride (11). The results obtained both with the photometric and Sullivan methods on solutions of these precipitates after removal of copper (cf. (1)) were consistently low (70 to 80 per cent) and these values are not reported.

Total S was determined by the Pregl method as described by Saschek (12), except that the BaSO\(_4\) was ignited. Up to 60 mg. of protein, distributed between two boats, can be burned in the Pregl apparatus without difficulty. About 1.5 cc. of Pregl’s peroxide solution are used on the spiral and the combustion is allowed to proceed slowly for 1 to 2 hours.

\(^1\) In some experiments, the CO\(_2\) passed from the HCl hydrolysate into an absorber containing a 20 per cent solution of CdCl\(_2\), but there was never any evidence of H\(_2\)S formation.

\(^2\) The 10 second interval was originally recommended by Sullivan (6). Later Rossouw and Wilken-Jorden (7) studied the timing of the Sullivan reaction in detail.
A direct determination of —SH groups in the unhydrolyzed protein (cf. (13-15)) was carried out only with reduced lactalbumin. The protein was treated with a large excess of dithiodiglycolic acid at pH 4.0 and room temperature, being stirred with N₂ for 10 to 15 hours; the thioglycolic acid formed was determined photometrically (1, 2) after precipitation of the protein by trichloroacetic acid.

The results are reported in Tables I and II.

**Table I**

*Effect of Hydrolysis on Cystine Determinations*

The values are the average of determinations on two to four hydrolysates.

<table>
<thead>
<tr>
<th>Hydrolysis</th>
<th>Lactalbumin</th>
<th>Edestin cystine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>Time</td>
<td>Photometric method</td>
</tr>
<tr>
<td>HCl</td>
<td>hrs.</td>
<td>per cent</td>
</tr>
<tr>
<td>6 * 0.2 N HCl</td>
<td>4</td>
<td>2.87</td>
</tr>
<tr>
<td>6 * 0.5 N HCl</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>6 * 0.1 N HCl</td>
<td>8</td>
<td>0.27</td>
</tr>
<tr>
<td>6 * 0.01 N HCl</td>
<td>15</td>
<td>0.23</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>48</td>
<td>2.87</td>
</tr>
<tr>
<td>20% HCl in 42% HCOOH</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>6 * 0.5 N HCl</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

* Micromodification.
† Hydrolysate strongly colored; values therefore too high.
‡ Approximately 1.0 per cent of cystine was found by the Sullivan method in cuprous chloride precipitates of the same hydrolysates.

**Humin Formation**—Humin formation during hydrolysis is frequently associated with losses of sulfur. For edestin, gliadin, and wool, Bailey (16) found that none of the methionine S is retained by the humin. On the other hand, Lugg (17) has shown that serious losses of cysteine occur owing to humin formation, while cystine is hardly affected. It therefore seems justified to correct the cysteine values found by the photometric method for the
Distribution of Sulfur

S retained by the humin. For this purpose, a sulfur determination according to Pregl's method is carried out on the thoroughly washed humin.

During HI hydrolysis there is no loss of cysteine due to humin formation. Therefore the values for cystine plus cysteine obtained by the Baernstein method are essentially correct (except for slight losses (1 to 2 per cent) due to decomposition of cysteine to H$_2$S).

With the proteins so far investigated the decomposition does not exceed 2 per cent, except with crystalline insulin, in which about 7 per cent of the cystine S is decomposed (cf. (3, 18)).

**Table II**

*Sulfur Amino Acids and Distribution of Sulfur in Various Proteins*

The values are given in per cent. Those given in parentheses are too high (see text).

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Substance and method of analysis</th>
<th>Casein (Labco)</th>
<th>Lactalbumin (Labco)</th>
<th>Reduced lactalbumin</th>
<th>Edestin</th>
<th>Papain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cystine, photometric</td>
<td>0.34*</td>
<td>2.80*</td>
<td>0.53†</td>
<td>1.2†</td>
<td>3.2*</td>
</tr>
<tr>
<td>2</td>
<td>Cysteine, &quot;</td>
<td>0.00*</td>
<td>0.27*</td>
<td>2.5†</td>
<td>0.00†</td>
<td>1.0†</td>
</tr>
<tr>
<td>3</td>
<td>Cystine, Sullivan</td>
<td>3.1*</td>
<td>(4.0)†</td>
<td>1.2†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Cystine + cysteine, Baernstein</td>
<td>0.48</td>
<td>3.1</td>
<td>3.2</td>
<td>1.5</td>
<td>4.2</td>
</tr>
<tr>
<td>5</td>
<td>Methionine, volatile iodide</td>
<td>3.2</td>
<td>2.8</td>
<td>2.7</td>
<td>2.4</td>
<td>0.46</td>
</tr>
<tr>
<td>6</td>
<td>Methionine, homocysteine</td>
<td>3.0</td>
<td>2.8</td>
<td>2.3</td>
<td>2.3</td>
<td>0.41</td>
</tr>
<tr>
<td>7</td>
<td>Cystine S</td>
<td>0.09</td>
<td>0.75</td>
<td>0.14</td>
<td>0.39</td>
<td>0.85</td>
</tr>
<tr>
<td>8</td>
<td>Cysteine S</td>
<td>0.00</td>
<td>0.07</td>
<td>0.07</td>
<td>0.00</td>
<td>0.27</td>
</tr>
<tr>
<td>9</td>
<td>Methionine S</td>
<td>0.69</td>
<td>0.60</td>
<td>0.58</td>
<td>0.51</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
<td>Sulfate S as H$_2$S</td>
<td>0.01</td>
<td>0.01 (0.05)§</td>
<td>0.02</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&quot; &quot; &quot; BaSO$_4$</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.54</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Total S, Pregl</td>
<td>0.78‖</td>
<td>1.42</td>
<td>1.43</td>
<td>0.98</td>
<td>3.01</td>
</tr>
<tr>
<td>13</td>
<td>&quot; &quot; 7 + 8 + 9 + (10, 11)</td>
<td>0.79</td>
<td>1.43</td>
<td>1.44</td>
<td>0.91</td>
<td>2.76</td>
</tr>
</tbody>
</table>

* 8 hour hydrolysis.
† 6 hour hydrolysis.
‡ Corrected (cf. (1)).
§ The H$_2$S is due to traces of thioglycolic acid which is decomposed to about 80 per cent during HI digestion (Brand, E., and Kassell, H., unpublished experiments).
‖ Total S was also determined with the Parr bomb.
A discrepancy between the photometric method and the Baernstein method is to be expected, if a protein on HCl hydrolysis yields considerable amounts of both cysteine and humin.

**Lactalbumin**—The lactalbumin (Labeo 7-HAAX) was obtained through the courtesy of Dr. G. C. Supplee.

In the hydrolysis of lactalbumin only moderate amounts of humin are formed. The sulfur retained by the humin is negligible (the humin formed during the HCl hydrolysis (16 hours at 130°) of 3.2 gm. of lactalbumin contained only 0.004 per cent of S). Table I shows that this protein contained only very small amounts of cysteine.4

The optimum time for HCl hydrolysis is 8 hours, since the results by the photometric, Sullivan, and Baernstein methods check (Tables I and II). Digestion for less than 8 hours results in incomplete hydrolysis, as indicated by the low cystine values. Longer hydrolysis also gives low results, owing partly to cystine destruction (cf. photometric values) and partly to increased formation of substances which interfere with the Sullivan method.

Hydrolysis with H₂SO₄ gives low results, particularly with the Sullivan method. This is apparently not due to cystine destruction, but to a tendency of the H₂SO₄ to produce substances which interfere with this reaction (cf. "Edestin").

On hydrolysis with the HCl-formic acid mixture, cystine destruction seems to be somewhat decreased, in agreement with the findings of Miller and du Vigneaud (4), but the rate of hydrolysis is also decreased (cf. Table I).

All of the sulfur in this preparation of lactalbumin is accounted for as cystine, cysteine, and methionine (Tables II and III). A preparation of lactalbumin analyzed by Baernstein (19) apparently contained small amounts of inorganic sulfate, but he likewise accounted for all of the protein sulfur as cystine and methionine.5

**Reduced Lactalbumin**—Reduced lactalbumin was prepared by treatment of lactalbumin with thioglycolic acid as described previously (21). The analysis reported in Table II refers to the

---

4 Another preparation of lactalbumin (used for feeding experiments in cystinuria) contained no cysteine.

5 Baernstein's preparation (19) contained more cystine and less methionine than ours. Plummer and Lowndes (20) analyzed a number of preparations of cow and of human lactalbumin and found variations in the total N, total S, methionine, and cystine. They did not account for the total S.
preparation of reduced lactalbumin used in feeding experiments in cystinuria (21).

With HCl the hydrolysis of the reduced protein seems to proceed somewhat faster than that of lactalbumin, since the same results were obtained after 6 and 8 hours (cf. (22)).

Hydrolysis with an HCl-formic acid mixture was not very satisfactory, because of the necessary evaporation of the formic acid. Since reduced lactalbumin contained large amounts of cysteine, HCl hydrolysis resulted in some loss (about 10 per cent of the cysteine) due to humin formation. This loss, indicated by the discrepancy between the values obtained after HCl and HI hydrolysis (cf. "Lactalbumin") could be confirmed by direct deter-

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total protein S*</th>
<th>Protein S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cystine</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Casein...........</td>
<td>0.78</td>
<td>12</td>
</tr>
<tr>
<td>Lactalbumin......</td>
<td>1.42</td>
<td>53</td>
</tr>
<tr>
<td>Reduced lactalbumin</td>
<td>1.38</td>
<td>10</td>
</tr>
<tr>
<td>Edestin...........</td>
<td>0.97</td>
<td>40</td>
</tr>
<tr>
<td>Papain............</td>
<td>1.47†</td>
<td>58</td>
</tr>
</tbody>
</table>

* Corrected for sulfate S.
† The possibility of the presence of organic sulfate S is not excluded.

The values for "cystine" found by the Sullivan method were too high (reported in parentheses in Table II), varying from 4.0 to 4.3 per cent for preparations of reduced lactalbumin in which about 80 per cent of the original cystine (3.1 per cent) had been reduced.

Attempts to determine cystine plus cysteine as total cysteine
by the Sullivan method after reduction with zinc or titanous chloride (cf. (22)) were not successful, owing to difficulties in removing the reducing agent.

The total sulfur and H₂S sulfur (Table II) in reduced lactalbumin are slightly higher than in lactalbumin. This is apparently due to traces of thioglycolic acid, which is decomposed to H₂S during digestion with HI (to an extent of about 80 per cent⁶). As in lactalbumin, all of the protein S of reduced lactalbumin is accounted for as cystine, cysteine, and methionine (Tables II and III).

**Casein**—Casein (Labsn) was obtained through the courtesy of Dr. G. C. Supplee. In view of its low cystine content, no detailed experiments on the effect of HCl hydrolysis were carried out. The casein contained no cysteine, and the results for cystine by the photometric and Baernstein methods are in reasonable agreement (Table II), although the value for cystine by the Baernstein method may be slightly high, owing to the possibility of titrating small amounts of homocysteine as cysteine (cf. (3)). All of the S of casein is accounted for as cystine and methionine (Tables II and III).

**Edestin** (Hoffmann-La Roche)—Table I shows that the optimum time for HCl hydrolysis is only 6 hours; hydrolysis for 8 hours resulted in lower values by the Sullivan method.

After H₂SO₄ hydrolysis, the values by the Sullivan method were always considerably lower than by the photometric method. That this was due to the formation of interfering substances was clearly demonstrated by finding more cystine by the Sullivan method in the cuprous chloride precipitate (9) than in the original hydrolysate (cf. Table I,† foot-note).

The values for cystine by the Baernstein method were almost 25 per cent higher than those by the photometric method.⁷ This discrepancy⁸ remains unexplained and subject to further investigation.

⁶ Unpublished experiments with dithiodiglycolic acid.
⁷ The value for cystine by the photometric method is in agreement with that found by Mirsky and Anson (14).
⁸ Unpublished experiments point to cysteine sulfinic acid (HOOC—
CH(NH₂)·CH₂·SO₂H) as a substance possibly responsible for the discrepancy, since we find that this compound is quantitatively reduced and determined as cysteine in HI digests. Its presence in HCl hydrolysates has no influence on the photometric cystine determination.
Distribution of Sulfur

It can be seen from Tables II and III that the S in edestin is not accounted for by cystine, methionine, and sulfate, and that at least 7 per cent of the protein S remains undetermined (cf. (23)). Cystine destruction during HI digestion is excluded by the low value for H_2S sulfur, which is in agreement with the gravimetric sulfate determination.

Papain—Recent reports (24, 25) indicate that papain, as well as the related enzyme, ficin, is a protein. It was therefore thought desirable to determine the distribution of the S in papain, particularly since no such data are available in the literature. The enzyme was a purified, water-soluble preparation of considerable activity, 1 mg. containing about 5 units (26).

The total S content was 3 per cent, of which about half was sulfate. The preparation contained about 3 per cent of cystine and 1 per cent of cysteine. (The values by the photometric and Baernstein methods checked.) There was a small amount of methionine present, which was determined both as volatile iodide and as homocysteine.

It can be seen from Tables II and III that the S in this preparation of papain is not accounted for by cystine, cysteine, methionine, and sulfate. About 17 per cent of the protein S (cf. Table III, foot-note) remains undetermined.

SUMMARY

1. In determining the sulfur distribution of a protein, a number of methods are combined in the following system of analysis. Total sulfur is determined by the Pregl method or in a Parr bomb. Sulfate is determined gravimetrically after hydrolysis with HCl, and as H_2S after digestion of the protein with HI. Methionine is determined as volatile iodide and as homocysteine. The sum of cystine and cysteine is determined in an HI digest. Cystine and cysteine are determined separately in an HCl hydrolysate by the photometric method. The cysteine value is corrected for the humin sulfur, determined by the Pregl method. The more specific, but less precise, Sullivan method is used as a check on the cystine determination.

Obtained through the courtesy of the Nippon Ferment Industrial Company.
2. Optimum conditions for HCl hydrolysis vary for different proteins. \( \text{H}_2\text{SO}_4 \) hydrolysis is not recommended.

3. The regular Sullivan method can be used for cystine determinations in proteins, only if the absence of cysteine in the hydrolysate has been established.

4. All of the sulfur of casein, lactalbumin, and reduced lactalbumin can be accounted for as cystine, cysteine, and methionine, while in edestin and in a preparation of papain considerable amounts of the sulfur remain undetermined.

BIBLIOGRAPHY

THE DISTRIBUTION OF THE SULFUR IN CASEIN, LACTALBUMIN, EDESTIN, AND PAPAIN
Beatrice Kassell and Erwin Brand


Access the most updated version of this article at http://www.jbc.org/content/125/2/435.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/125/2/435.citation.full.html#ref-list-1