Physical and Chemical Studies of a Limited Reaction of Iodine with Proteins*

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Although iodine is generally regarded as having two main effects on proteins, oxidation of sulfhydryl groups and substitution on the conjugated aromatic rings of tyrosine and histidine, conditions have been known for many years that apparently permitted only the oxidative reactions to occur (1-3). Investigations carried out under these conditions have generally been interpreted as suggesting oxidation to the level of disulfide, though sulfenyl iodide and higher states of oxidation of sulfur have been reported (4, 5). The structural implications of this postulated creation of new disulfide bonds in proteins led us to investigate the effects of treatment with small quantities of iodine upon the physical behavior of ovalbumin, $\beta$-lactoglobulin, and serum albumin. It would appear now that sulfenyl iodide formation is more common than previously supposed, that intermolecular disulfide formation does not occur with these proteins, and that intramolecular disulfide formation is not a major factor, though it cannot be completely excluded. Stoichiometry of the reaction as it relates to iodine-iodide-protein-substituted iodine is complicated by the appreciable spontaneous hydrolysis of the sulfenyl iodide group.

**EXPERIMENTAL**

**Spectrophotometric Rate Studies**—The extinction of the triiodide ion at 355 $\mu$m was employed in all experiments as a measure of iodine concentration. Standard curves were prepared by dilution of iodine solutions which had been standardized against National Bureau of Standards arsenious oxide by the usual procedure. All measurements were made in a Beckman DU spectrophotometer at $5^\circ \pm 1^\circ$. In solutions containing 0.2 M KI, the iodine concentration is related to the extinction at 355 $\mu$m by the equation, m.eq. I ml.$^{-1}$ = 7.77 x $10^{-5}$ x O.D.

The proper amount of sulfhydryl compound dissolved in 2.5 ml. of water for the rate experiments was obtained by dividing 0.00015 m mole by the number of equivalents of iodine expected to be consumed by the reaction. Sodium phosphate buffer, 0.5 ml. of 1 M, pH 6.5, was added to 2.5 ml. of the solution of sulfhydryl compound. Then 1.5 ml. of freshly prepared 0.6 M KI was added to give a total volume of 4.5 ml. A 3-ml. aliquot of the mixture was immediately placed in a quartz cuvette and cooled to 5$^\circ$ in the spectrophotometer. Two blanks containing water rather than sulfhydryl compound were always prepared simultaneously. After an initial reading to determine the very small control absorption at 355 $\mu$m, 0.2 ml. of standard iodine solution in 0.2 M KI was added to the sample, and also to one of the blanks to serve as a check on the concentration and stability of the standard iodine. The iodine concentration usually employed was near 0.001 M which corresponds to 0.0002 m.eq. of I in 0.2 ml. The sample was quickly mixed and timing begun. Measurements of the extinction were then made as a function of time. Direct sunlight was excluded from all solutions.

**Spectrophotometric Titrations**—Titrations of sulfhydryl compounds were performed under conditions essentially identical to those described previously for rate studies. The scale, and therefore the concentrations of the reagents, varied considerably in different experiments. The standard solution of iodine in iodide was, however, added in small quantities to the buffered solution of sulfhydryl compound in 0.2 M KI. The temperature was held between 0 and 5$. After each addition, a 3-ml. aliquot was removed and placed in the spectrophotometer. The extinction at 355 $\mu$m was then followed until it became constant or until its decrease became small and linear. This equilibrium value was plotted as a function of the quantity of iodine added.

**Optical Rotation Measurements**—These determinations were made in a 2-dm. cell thermostatted at 30$, in a Schmidt and Haensch instrument. Illumination was provided by a sodium vapor lamp.

**Ultracentrifuge Measurements**—Studies of the sedimentation characteristics of the proteins under investigation were made in the Beckman Spinco model B instrument. All runs were made at 59,780 r.p.m. Plate measurements were made with a Gaertner comparator.

**Materials**—Ovalbumin was prepared by the method of Warner (6). After three recrystallizations, the protein was dialyzed and lyophilized. The concentrations of ovalbumin solution were determined by the use of the relation, mg. of protein ml.$^{-1}$ = 1.36 x O.D. at 280 $\mu$m. $\beta$-Lactoglobulin was a commercial product of the Nutritional Biochemicals Company. Ultracentrifugal study of this material indicated it to be normal $\beta$-lactoglobulin (7) containing both A and B components in approximately the usual proportions. Concentrations of $\beta$-lactoglobulin solutions were determined with the use of the relation, mg. of protein ml.$^{-1}$ = 1.11 x O.D. at 280 $\mu$m. Crystalline bovine serum albumin was obtained from Armour Laboratories, and from Penex, Inc. Mercaptalbumin and bovine $\gamma$-globulin, fraction II, were obtained from Penex, Inc. Trypsin, chymotrypsinogen, $\alpha$-chymotrypsin, ovomucoid, yeast alcohol dehydrogenase, and lysozyme were products of the Worthington Biochemical Corporation. Human $\gamma$-globulin (fraction II) was generously supplied by the Cutter Laboratories. Crystalline...
inulin was a product of Eli Lilly and Company. Ribonuclease was obtained from the Sigma Chemical Company. All other compounds employed were commercial products of reagent grade or the equivalent.

**RESULTS**

**Rate and Extent of Reaction of Iodine with Representative Amino Acids and Peptides**—In order to establish the specificity of the iodine reaction under the exact conditions described previously, a series of amino acids was studied. Cysteine, as expected, reacted with 1 equivalent of iodine to form cystine. The rate curve is shown in Fig. 1. It was found, however, that if cysteine were allowed to stand in the presence of iodide at room temperature for more than 2 to 5 minutes before cooling and adding iodine, considerably less iodine was taken up. The results obtained with other compounds are listed in Table I. The sulfhydryl compounds tested reacted quite rapidly and the final iodine concentration was constant. The other compounds which reacted, however, did not give such sharp end points. An example is included in Fig. 1 for the case of tryptophan. The full extent of reaction of these compounds as listed in Table I is therefore only approximate. In general, specificity of the reaction for sulfhydryl groups in the case of proteins was substantiated. The interesting behavior of tryptophan and its derivatives was the only exception found. Presumably tryptophan in a protein would not interfere unless it occupied an N-terminal position.

**Rate and Extent of Reaction of Iodine with Various Proteins**—In the next series of measurements, proteins of varying sulfhydryl content were examined. In general the disappearance of iodine never completely stopped so that the equivalence point was taken as the point at which iodine disappearance reached a constant rate. An examination of representative rate curves in Fig. 2 will indicate the relatively small error introduced by this procedure in most cases. The results of these measurements are listed in Table II. If the reaction is run at pH 7.5 or at 20°, similar data are obtained, with the exception that the terminal slope is considerably greater in all cases. No protein known to be devoid of sulfhydryl groups reacted with iodine to any appreciable extent with the possible exceptions of chymotrypsin and γ-globulin. Ovalbumin and β-lactoglobulin react rapidly in a manner which yields a rather well defined end point. Reaction with bovine serum albumin or mercaptalbumin occurs in two distinct steps as illustrated in Fig. 2. Bovine and human γ-globulin react more slowly and since the uptake is small on a molar basis, the stoichiometry indicated must be considered to be approximate.

**Titration of Sulfhydryl Compounds with Iodine**—Since the possibility existed that the extent of reaction between iodine and sulfhydryl groups, particularly in proteins, might be different if iodine were not present in excess throughout the reaction, spectrophotometric titrations of several proteins, cysteine, and mercaptoethanol were performed. As indicated earlier, the residual extinction at 355 μm following the stepwise addition of iodine was measured. It was, of course, expected that no permanent

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**Table I**

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Initial Iodine</th>
<th>Final Iodine</th>
<th>Utilized</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m.eq × 10⁻⁶</td>
<td>m.eq × 10⁻⁶</td>
<td>m.eq/m.eq</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.00</td>
<td>2.03</td>
<td>1.03</td>
<td>0.97</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>1.78</td>
<td>2.19</td>
<td>0.41</td>
<td>0.22</td>
</tr>
<tr>
<td>ATR</td>
<td>1.46</td>
<td>2.05</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td>TrEE</td>
<td>4.74</td>
<td>2.05</td>
<td>2.72</td>
<td>0.57</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.20</td>
<td>1.40</td>
<td>1.20</td>
<td>6.00</td>
</tr>
<tr>
<td>Proline</td>
<td>145.0</td>
<td>2.10</td>
<td>1.86</td>
<td>0.92</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>88.6</td>
<td>2.10</td>
<td>0.60</td>
<td>0.03</td>
</tr>
<tr>
<td>Glycopeptide†</td>
<td>1.85</td>
<td>2.10</td>
<td>0.50</td>
<td>0.27</td>
</tr>
<tr>
<td>Histidine</td>
<td>92.2</td>
<td>2.03</td>
<td>0.93</td>
<td>0.20</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>86.7</td>
<td>2.00</td>
<td>0.95</td>
<td>0.20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.42</td>
<td>2.06</td>
<td>2.36</td>
<td>0.50</td>
</tr>
<tr>
<td>CMB</td>
<td>0.72</td>
<td>0.90</td>
<td>0.18</td>
<td>1.64</td>
</tr>
</tbody>
</table>

* The abbreviations used are: ATR, acetyl-L-tryptophan ethyl ester; ATR, acetyl-L-tryptophan; TrEE, L-tryptophan ethyl ester; CMB, p-chloromercuribenzoate.† Glycopeptide derived from ovalbumin (8); assumed mol. wt. = 1800.
optical density would be apparent until the equivalence point was reached, where an excess of I\textsuperscript{-} ion would be detected. It was found, however, that a residual extinction existed after only small additions of iodine to the proteins and that this color increased with further additions in a characteristic manner for each protein, until the sharp break associated with the equivalence point was observed. The behavior of ovalbumin and \(\beta\)-lactoglobulin is illustrated in Fig. 3. At the equivalence point ovalbumin has consumed 6.0 equivalents of iodine per mole and \(\beta\)-lactoglobulin, 3.8. With mercaptalbumin, the extinction at 355 \text{nm} continued to decrease slowly for long periods after addition of iodine. While this precluded the plotting of a well defined titration curve, a satisfactory equivalence point at about 2.1 equivalents of iodine per mole could be detected. These values are in good agreement with those obtained in the rate studies. Cysteine and mercaptoethanol titrated under the same conditions required 1 equivalent of iodine per mole as expected for conversion to the respective disulfides and no residual extinction at 355 \text{nm} was apparent until the equivalence point was reached.

Preparation of the Iodine-Treated Proteins—Preparations of iodine-treated proteins were made by performing titrations on a large scale. At the equivalence point the protein solutions were noticeably yellow. The solutions were then dialyzed exhaustively against distilled water in the cold and lyophilized. The yellow color faded rapidly during dialysis and the final solutions were practically colorless. Iodine analysis\textsuperscript{1} of the lyophilized proteins gave the following results: (a) Ovalbumin, 0.41 to 0.49 per cent; (b) \(\beta\)-lactoglobulin, none; (c) mercaptalbumin, none.

Comparison of Native and Iodine Treated Ovalbumin—Crystallization of iodine-treated ovalbumin could be accomplished by the procedure employed for ovalbumin itself though with some difficulty. The lyophilized material was used for all other studies. The specific rotation found for native ovalbumin in phosphate buffer at pH 7.5 and 30\textdegree Celsius was \(-31.1\)\textdegree (9) while for the iodine-treated ovalbumin it was \(-31.1\)\textdegree. The rate of denaturation of both proteins with urea was followed by measuring the change in optical rotation with time. The results are given in Fig. 4. At the end of each experiment the solutions were adjusted to pH 5 and diluted with 3 volumes of water. More than 92 per cent of the protein was insoluble in both cases. The susceptibility of this denatured protein to the action of trypsin was tested as described in an earlier publication (10). The denatured proteins were digested at identical rates and all susceptible bonds were found to be hydrolyzed, within the limits of accuracy of the determination.

Ultracentrifugal studies were made at several concentrations of ovalbumin and iodine-treated ovalbumin. In all cases the general shape of the sedimenting boundary and the measured sedimentation constants indicated no change in the physical characteristics of ovalbumin after iodine treatment. No evidence for high molecular weight polymers, which might be formed through intermolecular disulfide formation, could be obtained.

Ultracentrifugal Studies of Native and Iodine-Treated Lactoglobulin and Serum Albumin—Ultracentrifugal analysis of native and iodine-treated \(\beta\)-lactoglobulin under conditions where \(\beta\)-lactoglobulin behaves essentially as a single component indicated a complete identity of the native and treated proteins as to both sedimentation constant and degree of heterogeneity. The same was found with serum albumin, confirming the observation of Hughes and Straessle (5) made under somewhat different conditions.

Studies of Iodine-Treated Proteins Before Dialysis—The reaction of rather large amounts of iodine with ovalbumin and lactoglobulin together with the relatively small amounts of iodine determined to be combined with these proteins after dialysis and lyophilization would suggest that the major role of iodine is oxidative. However, the yellow color present in the protein solution just at the equivalence point is reminiscent of the stable

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Protein & Molecular weight & Iodine & I reacted & \textbf{Ratio} \\
& (\text{m} \cdot \text{eq.} \times 10^4) & (\text{m} \cdot \text{eq.} \times 10^4) & (\text{m} \cdot \text{eq.} \times 10^4) & \\
\hline
Ovalbumin & 0.382 & 45,000 & 2.02 & 0.55 & 2.34 & 6.12 \\
\(\beta\)-Lactoglobulin & 0.367 & 37,000 & 2.04 & 0.61 & 1.43 & 3.80 \\
\(\gamma\)-Globulin (bovine) & 0.254 & 176,000 & 2.08 & 1.92 & 0.16 & 0.631 \\
\(\gamma\)-Globulin (human) & & & & & & \\
Serum albumin (bovine) & 0.526 & 65,000 & 2.04 & 1.54 & 0.50 & 0.951 \\
Mercaptalbumin & 0.754 & 65,000 & 2.04 & 1.22 & 0.82 & 1.101 \\
\(\alpha\)-Chymotrypsin & 1.06 & 25,000 & 2.08 & 1.87 & 0.21 & 0.181 \\
Chymotrypsinogen (yeast) & 1.50 & 25,000 & 2.04 & 0.02 & 0.02 & 0.03 \\
Insulin & 4.38 & 6,000 & 2.04 & 1.86 & 0.18 & 0.041 \\
Lyszyme & 0.80 & 17,200 & 2.09 & 2.08 & 0.01 & 0.01 \\
Ovomucoid & 0.82 & 28,000 & 2.04 & 0.03 & 0.01 & 0.01 \\
Ribonuclease & 1.17 & 14,000 & 2.08 & 0.08 & 0.00 & 0 \\
Trypsin & 1.25 & 25,000 & 2.08 & 0.01 & 0.06 & 0.04 \\
Alcohol dehydrogenase (yeast) & 0.08 & 150,000 & 2.08 & 0.14 & 1.34 & 17.001 \\
\hline
\end{tabular}
\caption{Extent of Reaction of Iodine with Proteins at 5\textdegree C and at pH 6.5}
\end{table}

\textsuperscript{1} Iodine analyses were performed by the Clark Microanalytical Laboratory, Urbana, Illinois.

![Fig. 3. The titration of proteins with iodine at pH 6.5 and at 0-5\textdegree C. (O-O) \(\beta\)-lactoglobulin, 3.11 \times 10^{-4} \text{m} \text{mole} \text{ per ml}; (\Delta-\Delta) ovalbumin, 2.26 \times 10^{-4} \text{m} \text{mole} \text{ per ml. For other details, see text.}](http://www.jbc.org/)
p-Lactoglobulin was adjusted to pH 6.5 with NaOH. The actual buffer composition was 0.11 M Na$_2$HPO$_4$ and 0.2 M pH 7.5.

Total volume 18 ml. (O-O) no additional treatment; (O-O) treated with iodine at pH 6.5 and at 5°. 5.21 X 10$^{-4}$ mmoles of protein to which 3.8 equivalents of I per mole-r had been added.

Ovalbumin (●-●) and iodine-treated ovalbumin (O—O). Bovine serum albumin.

**TABLE III**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration</th>
<th>Buffer</th>
<th>$s_{20 \text{w}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>0.50</td>
<td>Tris,* pH 7.81</td>
<td>3.55</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>Tris,* pH 7.81</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>1.27</td>
<td>Tris,* pH 7.81</td>
<td>3.22</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>Tris,* pH 7.81</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td>0.72</td>
<td>Tris,* pH 7.81</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>1.51</td>
<td>Tris,* pH 7.81</td>
<td>3.25</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>1.5</td>
<td>Tris,* pH 7.81</td>
<td>2.74</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>1.5</td>
<td>Tris,* pH 7.81</td>
<td>2.81</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.85</td>
<td>Phosphate, KI</td>
<td>3.87†</td>
</tr>
<tr>
<td></td>
<td>0.85</td>
<td>Phosphate, KI</td>
<td>3.90†</td>
</tr>
</tbody>
</table>

* Tris, tris(hydroxymethyl)aminomethane.
† These values are not corrected to standard conditions. The actual buffer composition was 0.11 m Na$_2$HPO$_4$ and 0.2 m KI adjusted to pH 6.5 with NaOH.

**Fig. 4.** The effect of urea upon the optical rotation of native ovalbumin (●—●) and iodine-treated ovalbumin (O—O). Urea concentration, 7.2 m; 30°; 0.035 m sodium phosphate buffer, pH 7.5.

**Fig. 5.** The stability of the residual color of β-lactoglobulin treated with iodine at pH 6.5 and at 5°. 5.21 X 10$^{-4}$ mmoles of protein to which 3.8 equivalents of I per mole$^{-1}$ had been added. Total volume 18 ml. (●—●) no additional treatment; (O—O) after addition of 5.21 X 10$^{-4}$ mmoles of 2-mercaptoethanol.

sulfonyl iodide of tobacco mosaic virus described by Fraenkel-Conrat (4). At 5° the yellow color of iodine-treated ovalbumin and lactoglobulin fades slowly. If the temperature is raised to 20°, a greatly increased rate of discharge of color occurs. Treatment of the solution at 5° with a 100-fold molar excess of cysteine, 2-mercaptoprothamide, or 2-mercaptoethanol results in a rapid and complete loss of color. This is illustrated in Fig. 5, and it is to be expected if the color is indeed due to the presence of sulfonyl iodide groups in the protein, since such groups would react rapidly to yield mixed disulfides. This would explain the loss in color on prolonged dialysis during the preparative procedures, and the subsequent low analyses for iodine.

**DISCUSSION**

Studies of amino acids suggest that the reaction between iodine and proteins at low temperature and near neutral pH can be ascribed largely to the sulfhydryl content of the proteins, with the minor but interesting possible exception of N-terminal tryptophan. A correlation between the number of equivalents of iodine consumed and the number of sulfhydryl groups obtained by the application of other methods of measurement should then yield information as to the nature of the iodine sulfhydryl reaction.

The simplest case which we have investigated would appear to be that of β-lactoglobulin which consumes approximately 4 equivalents of iodine per mole (37,000). Formation of the residual yellow color proceeds uniformly throughout the titration to the equivalence point, and is relatively stable. β-Lactoglobulin has been reported to contain 2 sulfhydryl groups per mole on the basis of its binding of 4-(p-diethylaminobenzesazo)phenyl mercuric acetate (11). We have confirmed this figure through the use of p-chloromercuribenzoate by the method of Boyer (12). In addition, evidence has recently been presented which indicates that the true monomer weight of β-lactoglobulin is about 18,000 (13). This information, together with the behavior of the yellow color of the iodine-treated protein upon treatment with sulfhydryl compounds, suggests that the single sulfhydryl of the β-lactoglobulin monomer reacts with two equivalents of iodine to yield iodide and the sulfonyl iodide.

The case of ovalbumin is considerably more complex. The shape of the iodine-O.D. 355 m titration curve indicates the formation of two sulfenyl iodide groups (4 equivalents of iodine). However, 2 further equivalents of iodine are consumed in an unknown fashion, and in contrast with lactoglobulin, 1.4 to 1.7 equivalents of iodine per mole of protein are found to be bound to ovalbumin even after prolonged dialysis. All recent evidence indicates the presence of 4 sulfhydryl groups in ovalbumin (10-14). One of these appears to be masked to p-chloromercuribenzoate unless the protein is denatured. Of the remaining three, two have been reported to be somewhat more reactive toward organic mercurials than the third (11). One might presume that these two reactive sulfhydryl groups account for the formation of 2 moles of sulfenyl iodide. Oxidation of the two remaining sulfhydryl groups to an internal disulfide could account for the over-all stoichiometry. Intermolecular disulfide formation is ruled out by the demonstration that there is no change in sedimentation constant after iodine treatment. If an intramolecular disulfide bridge is formed it has remarkably little effect on the native structure of the molecule since the iodine-treated protein can be crystallized and exhibits an optical rotation very nearly identical to that of native ovalbumin. The rate of urea denaturation is somewhat greater in the case of the iodine-treated ovalbumin but the final rotation is close to that of the native protein.
found for untreated ovalbumin after urea denaturation. No effect on the susceptibility to trypsin of the lysine and arginine residues of iodine-treated ovalbumin could be detected. Preliminary chemical analyses of acid hydrolysates of the oxidized ovalbumin have not provided evidence for increased cystine content. The location of the protein-bound iodine in the ovalbumin preparation is obviously the key to a complete understanding of the stoichiometry of the reaction. Unfortunately it has not yet been possible to decide whether this represents relatively stable sulfenyl iodide which exhibits markedly different spectral characteristics, or substitution upon some other component of the molecule. In addition to the hydrolysis studies noted previously, other chemical and spectral investigations are currently being directed toward a resolution of this problem.

**SUMMARY**

1. A spectrophotometric method for the estimation of sulfhydryl groups based on the extinction of triiodide ion at 355 nm is described.

2. The application of this method to proteins containing sulfhydryl groups suggests the formation of protein sulfenyl iodide to be a common reaction under experimental conditions of neutral pH and low temperature.

3. β-Lactoglobulin reacts with 4 equivalents of iodine to form 2 moles of sulfenyl iodide per mole of protein (37,000). Ovalbumin reacts with 6 equivalents of iodine to form 2 moles of sulfenyl iodide per mole of protein (45,000). The nature of the reaction of the remaining 2 equivalents of iodine is unknown.

4. Intermolecular disulfide formation as an effect of iodine oxidation of these proteins under these conditions was ruled out by ultracentrifugal analysis. After dialysis and lyophilization the iodine-treated proteins exhibit properties quite similar to those of untreated samples.

**Acknowledgment**—The technical assistance of Miss Diane Karraker in the mercaptide formation studies of lactoglobulin is gratefully acknowledged.

**REFERENCES**

Physical and Chemical Studies of a Limited Reaction of Iodine with Proteins
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