THE REACTION OF TOBACCO MOSAIC VIRUS WITH IODINE*

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The action of iodine on proteins has generally been regarded as producing two main effects: (1) the oxidation of any —SH groups to disulfide bonds, and (2) the substitution of ring hydrogen atoms of tyrosine and histidine residues. It has been noted, however, that in certain instances, notably tobacco mosaic virus (TMV) and serum albumin (1, 2), 2 to 3 atoms of iodine are rapidly consumed by each —SH group, although only 1 would be needed for oxidation to the disulfide. This is indicative of a two-step oxidation, or a substitution reaction. Since aliphatic sulfenic acids or sulphenyl iodides, the products of such reactions, are notoriously unstable, this interpretation has been usually rejected. It now appears that sulphenyl iodide groups are actually produced and are stable in native TMV. This finding indicates that the presumably steric stabilization of the masked protein —SH group can be transferred to derivatives of this group.

EXPERIMENTAL

Reaction Conditions—Iodination was generally performed in 0.05 M phosphate buffer (pH 6.8) at room temperature or at 0°, by adding 0.01 to 0.05 N iodine solutions in a 10- to 50-fold concentration of potassium iodide (1). With less iodide, or with alcoholic iodine solutions, no unequivocal results were obtained. The amount of iodine used ranged from equivalence to 10-fold excess over the —SH groups of the protein. To terminate the reaction, unchanged iodine was titrated with 0.004 N thiosulfate. The protein was freed from iodide ions by long dialysis. In the case of TMV, repeated ultracentrifugal separations, ammonium sulfate precipitations, and dialysis were used to remove the last traces of iodide. When I^{131} was employed, radioactive iodide was removed by repeated displacement with chloride in the course of dialysis or ultracentrifugal separation. Ordinary iodide was also often used to wash out radioactive iodide ions.

Analytical Characterization of Products—The —SH content of proteins was determined by titration with N-ethyl maleimide after denaturation with guanidine salts, with nitroprusside as indicator (3). Similar results were obtained with guanidine bromide containing sodium carbonate and Versene (4), and also with acid-buffered guanidine chloride, followed by an

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alkaline buffer prior to addition of nitroprusside and titration (3); Versene was substituted for cyanide also in the latter test (5).

An additional –SH test often used was based on the spectrophotometric observation of the mercaptide formation upon addition of p-chloromercuribenzoate (6). This test was usually performed in 1 per cent sodium dodecyl sulfate (SDS) at pH 5 (0.1 to 0.2 M acetate) or at pH 7. The molar absorption increment (ΔεM) due to mercaptide formation as measured at pH 5 with cysteine or glutathione in 1 per cent SDS at 255 mμ was 7.8 × 10³, while, in the absence of SDS, Boyer’s value of 6.2 × 10³ was corroborated. Several hours were needed for the maximal increment to be reached in SDS, and calculations were based on the final readings, stable in the presence of an excess of at least 0.02 μmole of p-chloromercuribenzoate per ml. The interpretation of these assays was ambiguous when iodide was present or was liberated from the protein during the test, since this ion caused slightly greater spectral changes with the mercuribenzoate than an equivalent amount of –SH groups.

For cystine determinations, the proteins were hydrolyzed in evacuated (16 mm. of Hg) sealed tubes with redistilled 5.7 N hydrochloric acid for 10 hours at 105°. The hydrolysates were neutralized and analyzed according to Sullivan and Hess (7).

The amino N of proteins was determined by the ninhydrin procedure (5, 8). When I^{131} was used, the extent of iodination was determined on solutions in a well type scintillation counter. Analyses for I^{127} were performed by Mr. Tashinian of the Department of Chemistry.

Results

Reaction with Iodine—When a series of proteins containing –SH groups was treated at pH 6.8 with an amount of iodine equivalent to their –SH content, some consumed the iodine instantaneously, even at 0°, while other reaction mixtures became colorless only after a few minutes at 0° or at room temperature. A 2nd equivalent was usually used up at a somewhat slower rate. When appreciably more iodine was added, most proteins continued to react with it without reaching a definite end-point. TMV represented an exception, in that this nucleoprotein consumed in 10 to 15 minutes at room temperature about 2 equivalents of iodine but additional amounts at an almost negligible rate (about one-twentieth of that of the first reaction). The nitroprusside test in guanidine solution remained faintly positive until 2 atoms of iodine had reacted.

The apparent reaction of 2 atoms of iodine with one –SH group in the case of TMV (2.1 per 20 mg., i.e., 0.9 –SH group, is the extrapolated value) led to a search for a sulfenic acid or sulfenyl iodide group in the reaction product. The virus pellet, even after extensive purification, was yellow,
which is a characteristic of sulfenyl iodides (9). Iodine analyses indicated
that of the first 1 to 2 atoms of iodine added to TMV approximately one-


half remained bound by the protein. The end-product of the first reaction
(see above) contained from 0.75 to 0.95 per cent of iodine, while control


virus treated with iodide alone retained only negligible amounts (about


one-tenth as much) after the same purification. Results with I\textsuperscript{1131}
confirmed these observations. Of the first 1 to 10 iodine atoms added to the


virus no more than 2 reacted at an appreciable rate, and almost half of


this amount was bound by the virus (Table I). There appeared to be no
doubt that the first reaction, in the case of TMV, was a substitution and


that the \(-\text{SH}\) group was involved in the reaction. All other \(-\text{SH}\)
proteins bound only an insignificant fraction of the 1st iodine atom (I\textsuperscript{1131})
added, indicating that oxidation to the disulfide was the predominating
reaction in these cases.

When TMV was treated at pH 6.8 with iodine either in alcoholic solution


dissolved with a minimum of potassium iodide, more iodine was rapidly


bound, indicating a lesser differential in the reaction rates of the \(-\text{SH}\) and


phenolic groups under these conditions. The same was the case at a higher


pH.

Mode of Fixation of Iodine—Results discussed in the previous section


appear to permit only one conclusion: The reaction of the \(-\text{SH}\) group


of TMV with iodine is one of substitution and yields a sulfenyl iodide group


\((-\text{SH} + \text{I}_2 \rightarrow -\text{SI} + \text{HI})\). Since aliphatic sulfenyl iodides are known to


be extremely reactive (9, 10), the masked state of the original \(-\text{SH}\) group


must be invoked as an explanation for the survival of this group in the virus.
In this case, denaturation which unmasks the \(-\text{SH}\) group should also re-


store to the sulfenyl iodide group its "normal" reactivity. One of the re-


actions of sulfenyl iodides is that with mercaptans to yield disulfides.


Accordingly, iodo TMV\textsuperscript{1} was treated with known amounts (1.5 to 10 equiva-


lents) of cysteine or glutathione, followed by a denaturant. In agreement


with expectation, about 1 equivalent of the added thiol disappeared for
each original \(-\text{SH}\) group during this reaction (Formula I). In control


(I) TMV\(-\text{SI} + \text{HS}-\text{CH}_2-\text{CH(\text{NH}_2)-COOH} \xrightarrow{\text{denaturation}} \text{TMV-S-S-CH}_2-\text{CH(\text{NH}_2)-COOH} + \text{HI}


experiments with TMV, on the other hand, the expected \(-\text{SH}\) titer for the


sum of the protein and the added thiol was generally approximated. When


the protein was precipitated from such reaction mixtures, the supernatant


solutions from TMV contained almost all of the added \(-\text{SH}\) compound,


\textsuperscript{1} This abbreviation will be used for the purified reaction product of iodine with


TMV, showing a sulfur-iodine molar ratio of about 1.
### Table I

**Extent of Substitution and Stability of Bound Iodine in Proteins Treated with Small Amounts of Iodine**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reaction*</th>
<th>Substitution</th>
<th>SDS stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iodine atoms used per -SH group*</td>
<td>nH</td>
<td>Temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8</td>
<td>Room</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>4.6 (2.5)*</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.3</td>
<td>0</td>
</tr>
<tr>
<td>TMV protein Preparation A</td>
<td>2.9</td>
<td>6.8</td>
<td>Room</td>
</tr>
<tr>
<td>TMV protein Preparation B</td>
<td>1.3</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turnip yellow mosaic virus</td>
<td>1</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td>Tomato bushy stunt virus</td>
<td>2</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>1</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mercaptalbumin</td>
<td>1</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10</td>
<td>0</td>
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<tr>
<td>β-Lactoglobulin</td>
<td>1</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td>Papain</td>
<td>1</td>
<td>6.8</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.5</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>6.8</td>
<td>0</td>
</tr>
</tbody>
</table>

* Added iodine (I\(^{131}\)) was used up in 1 to 2 hours except when TMV was treated with 4.6 equivalents, in which case 2.1 equivalents were titrated after 1 hour at room temperature (20–23°C). The rate of discharge of the iodine color is indicated by the words slow (10 minutes or more), fast, instantaneous.
**Table I—Concluded**

† Calculated as per cent of maximum, had all the added iodine reacted by substitution and none by oxidation (i.e., half of that added would be bound). The figures in parentheses indicate the number of experiments averaged.

‡ Calculated as per cent of the originally bound iodine retained by the protein after treatment with 1 per cent sodium dodecyl sulfate (SDS) at 40° and pH 7 for 24 hours, followed by exhaustive dialysis.

§ TMV treated with iodine at pH 5 and pH 9 to 10 was largely insoluble and retained much iodine by occlusion. When redissolved with alkali and ultracentrifuged, protein fractions were obtained in poor yield, which contained the indicated amounts of iodine.

∥ This protein contains no --SH groups. The extent of reaction is expressed in terms of the sulfur atom or subunit weight, respectively.

¶ The --SH content of papain and serum albumin appears to be variable and more difficult to determine accurately than that of the other proteins listed. Thus the I to SH ratio is more in doubt.

**Table II**

---SH Groups and Thiol-Binding Capacity of Iodo TMV and TMV---

<table>
<thead>
<tr>
<th>Conditions of denaturation and test</th>
<th>µeq. per 20 mg. air-dry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iodo TMV</td>
</tr>
<tr>
<td></td>
<td>--SH</td>
</tr>
<tr>
<td>Guanidine, HBr (pH 11); N-ethyl maleimide†</td>
<td>0.00</td>
</tr>
<tr>
<td>Guanidine, HCl (pH 4.8); N-ethyl maleimide (pH 9.7)</td>
<td>0.00</td>
</tr>
<tr>
<td>SDS (pH 5.0); p-chloromercuribenzoate§</td>
<td>(1.35)¶</td>
</tr>
<tr>
<td>SDS (pH 5.0); guanidine, HBr (pH 11); N-ethyl maleimide</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Averages of two to seven analyses, agreeing, except for isolated cases, within 10 per cent. The added thiol was cysteine or glutathione, usually 2 to 5 µeq. per ml. of a 2 per cent virus solution.

† Tsao and Bailey (3).

‡ Denaturation (i.e. clearing) by 1 per cent SDS at pH 5.0 required several days at room temperature or 1 to 2 hours at 40°, followed by standing at room temperature for 16 hours. The reaction mixtures contained 0.002 M Versene. Aliquots (about 1.5 to 2 mg. of virus) were diluted with the same solvent mixture to 4 ml. for the spectrophotometric p-chloromercuribenzoate reaction, while other aliquots (5 to 15 mg. of protein) were treated with twice the volume of the guanidine solution prior to titration with N-ethyl maleimide.

§ Boyer (6).

¶ Spurious analysis, due to liberated iodide reacting with p-chloromercuribenzoate (average of fourteen analyses ranging from 1.2 to 1.6).
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while in those from iodo TMV there was a deficit of about 1 equivalent. These experiments were performed with various combinations of denaturants and -SH tests, as illustrated by a few examples in Table II.

It appeared important to prove the actual incorporation of added cysteine by disulfide linkage to the protein. To this end, the denatured protein was isolated and analyzed for cystine after hydrolysis. Considerable variations were observed in the cystine content of TMV, depending upon the technique of preparation of the sample. After precipitation of the virus protein from guanidine solution with water, the hydrolysates were almost colorless and the cystine content was, within the expected range, 0.65 to 0.8 per cent. In contrast, nucleoprotein preparations obtained after SDS treatment and dialysis and hydrolyzed by the same technique (in sealed evacuated tubes at 105° for 10 hours) gave dark hydrolysates, of low and variable cystine content (0.35 to 0.5 per cent). No better results were obtained after hydrolysis in the presence of titanous chloride (11).

When iodo TMV was analyzed in the same manner, this preparation yielded similar cystine values, i.e. about 0.65 and 0.4 per cent, depending on the technique of preparation. However, samples treated with cysteine in the presence of guanidine salts or SDS yielded cystine values which were usually about 30 per cent higher than the corresponding control preparations. Thus the postulated reaction mechanism (Formula I) appears qualitatively established.

Further evidence for the incorporation of half cystine residues came from amino N analyses of the same preparations. As expected, those derived from iodo TMV denatured in the presence of cysteine or glutathione were

<table>
<thead>
<tr>
<th>After treatment with</th>
<th>Amino N of Nucleoprotein Isolated after Dialysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iodo TMV†</td>
</tr>
<tr>
<td></td>
<td>TMV†</td>
</tr>
<tr>
<td>Cysteine, SDS, pH 5</td>
<td>0.136</td>
</tr>
<tr>
<td>No cysteine, SDS, pH 5</td>
<td>0.117</td>
</tr>
<tr>
<td>Cysteine, SDS, pH 7</td>
<td>0.145</td>
</tr>
<tr>
<td>No cysteine, SDS, pH 7</td>
<td>0.120</td>
</tr>
</tbody>
</table>

* The analyses (8) are averages of about ten experiments at pH 5, and three experiments at pH 7. The range of most of the averaged values was ±0.005 per cent NH₄-N.
† It must be noted that these analyses were performed with the denatured and dialyzed reaction products; thus iodo TMV signifies the preparation derived from iodo TMV, but retaining at this stage only traces of iodine.
definitely and consistently higher than the controls (Table III). However, this effect was never as great as that expected, i.e. 50 per cent increase in amino N, nor did the cystine analyses referred to above indicate quantitative reaction. Disulfide exchange between the substituted protein and the excess cysteine in the reaction mixture to yield protein disulfide dimer and free cystine would supply an explanation for the observed deficits in bound half cystine and amino groups. A few preliminary analyses for cystine in the presence of cysteine have indicated that iodo TMV actually stimulated such an over-all oxidation under certain conditions.

Denaturation caused the loss of much of the bound iodine (or I\textsubscript{131}) also in the absence of —SH compounds (Table I). In this case, hydrolysis followed by dismutation of the sulfenic acid, or more probably condensation with nitrogenous groups of the protein, might be involved. The lowered amino N of the protein isolated after denaturation from iodo TMV, as contrasted with the protein from TMV, appears to support this hypothesis (Table III). However, added lysine was not fixed through a sulfenyl amide linkage to the protein during the denaturation of iodo TMV, as judged by the amino N of the dialyzed preparation. The nature of the degradation product of the sulfenyl iodide group formed during denaturation thus remains obscure. The fact that it reverts to cystine during hydrolysis appears significant. It must also be noted that some capacity to bind or oxidize cysteine is retained by the product for several hours under the conditions of denaturation.

In contrast to its lability upon denaturation, the sulfenyl iodide group of the undenatured iodo TMV appeared quite unchanged after several weeks storage in the refrigerator, and its I\textsubscript{131} content was not appreciably diminished during prolonged washing and equilibration with KI\textsubscript{197}.

Proteins other than TMV bound an appreciable fraction of the added iodine only upon treatment with amounts in excess of those equivalent to their —SH groups; all iodine was bound in such a manner that it was not released by denaturation. This conforms with the expected stability of iodo-phenyl residues (Table I).

The results obtained with two protein preparations derived from TMV were of particular interest. A low molecular weight preparation which was obtained from the virus by treatment with detergent (12) and which had lost its —SH groups, presumably through autoxidation in the course of isolation (Preparation A), resembled other sulfhydryl-free proteins, e.g. insulin. This protein bound close to one-half of the added iodine, although slowly, and none was released by denaturation. Thus all of the iodine was presumably present as diiodotyrosine.

In contrast, a sulfhydryl-containing protein (Preparation B), obtained by ammonium sulfate precipitation after treatment with carbonate-bicar-
bonate buffer at pH 10.5 and 3° for 48 hours, reacted even more slowly with iodine and bound none of the 1st and only a small fraction of the next 2 to 4 atoms of added iodine. The part that was bound was stable to denaturation. The smallest amount of iodine added sufficed to abolish the —SH group. It thus appears that the —SH group of this protein fraction from TMV is not sufficiently masked to form a stable sulfenyl iodide group.

DISCUSSION

It has been observed that, under certain conditions, iodine transforms the —SH groups of TMV not to disulfide but to sulfenyl iodide groups. This reaction can be more readily envisaged than oxidation to disulfide bridges when one is dealing with a macromolecule composed of a fixed arrangement of subunits, each containing a single —SH group. Also, the retention of biological activity (1)² of such an oxidation product appears less surprising than it would be for a structure with artificial disulfide cross-links. On the other hand, it must be recognized that aliphatic sulfenyl iodides are generally regarded as too unstable³ to represent final reaction products. That they play such a rôle in the case of TMV may be an indication of an as yet unrecognized ability or function of proteins. Proteins, apparently, can protect and preserve normally unstable reaction intermediates within their hydrogen-bonded lattice structure. Indications have been obtained that this is possible also with a simpler protein and with a reactive group unrelated to sulfenyl iodide.

A survey of a number of —SH-containing proteins, including other viruses, did not yield another case in which a stoichiometric amount of a stable sulfenyl iodide group was formed. In all of these proteins, only about 1 atom of iodine was needed to abolish the —SH groups,⁴ and none or little (less than one-quarter) of the added iodine was bound under these conditions. The iodine which was bound by these proteins was not dislodged by denaturation and thus appeared to be situated on phenol or imidazole rings.

The present findings may have general significance beyond the demonstration that proteins may stabilize and preserve highly reactive intermediates which may well play a rôle in enzymatic reactions. The observed formation of sulfenyl iodide groups under the influence of iodine suggests

² The retention of viral activity by iodo TMV was confirmed in the course of the present study.

³ The only aliphatic sulfenyl iodide that has been prepared (9), derived from tertiary butyl mercaptan, decomposed slowly in ether solution in the cold and could not be isolated in analytically pure state.

⁴ In the case of mercaptalbumin, these results are contrary to expectation, based on the findings of others (2) under somewhat different conditions. No appreciable dimerization occurred (personal communication of Dr. H. K. Schachman), and no satisfactory interpretation of the data appears possible at this time.
a more general hypothesis for the mechanism of oxidation of \(-\text{SH}\) groups, even in cases in which the disulfide group is the only reaction end-product. Thus a formulation based on the assumption of two consecutive bimolecular reactions, rather than one termolecular reaction, appears now preferable (Formulas II, a and b compared to Formulas III, a and b), even though the reactive intermediate can in most instances not be demonstrated.

\[
\begin{align*}
(\text{II, a}) & \quad \text{R}--\text{SH} + \text{I}_2 \rightarrow \text{R}--\text{SI} + \text{HI} + \text{R}'\text{SH} \rightarrow \text{R}--\text{S}--\text{S}--\text{R'} + 2\text{HI} \\
(\text{II, b}) & \quad \text{R}--\text{SH} + \frac{1}{2}\text{O}_2 \rightarrow \text{R}--\text{SOH} + \text{R}'\text{SH} \rightarrow \text{R}--\text{S}--\text{S}--\text{R'} + \text{H}_2\text{O} \\
(\text{III, a}) & \quad \text{R}--\text{SH} + \text{R'}--\text{SH} + \text{I}_2 \rightarrow \text{R}--\text{S}--\text{S}--\text{R'} + 2\text{HI} \\
(\text{III, b}) & \quad \text{R}--\text{SH} + \text{R'}--\text{SH} + \frac{1}{2}\text{O}_2 \rightarrow \text{R}--\text{S}--\text{S}--\text{R'} + \text{H}_2\text{O}
\end{align*}
\]

Similar ideas have previously been expressed and discussed (13, 14), but this appears to be the first instance in which the sulfenic acid stage of oxidation has been experimentally demonstrated.

**SUMMARY**

1. Treatment of tobacco mosaic virus with iodine at neutrality transforms the sulphydryl to a stable sulfenyl iodide group.
2. Upon denaturation of the nucleoprotein, the sulfenyl iodide group acquires its typical reactivity and instability.
3. The \(-\text{SH}\) groups of other proteins are predominantly oxidized to disulfide groups under the same conditions.
4. Various implications of these findings are discussed.

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