Proteins may contain several actual or potential sulfhydryl groups. The importance of these groups for cellular respiration had been recognized early by de Rey Pailhade and has been since extensively investigated by Hopkins and his coworkers (11, 13, 14). Such groups appear to be of importance not alone for purely oxidation-reduction mechanisms but for the enhanced functioning of certain hydrolytic biocatalysts such as urease (10, 28), papain (4, 5), kathepsin (19), and cerebrosidase (33). On the other hand, the presence of intact disulfide linkages appears to be necessary to the physiological behavior of other active principles such as insulin (31, 35), the appearance of free —SH groups coinciding with loss in activity.

Proteins, other than the biocatalysts alluded to, such as hemoglobin, myosin, and the lens proteins, may contain in the native state free —SH groups, as the experiments of Todrick and Walker (34), Anson and Mirsky (1), and Mirsky (20) have demonstrated. The number of these free groups in these and many other proteins is increased by treatment of the protein with denaturing agents (20). Methods for estimating the concentration of sulfhydryl groups in native and denatured proteins have been devised by Todrick and Walker (34) and by Mirsky and Anson (21). These have been applied exclusively to heat-denatured or trichloroacetic acid-precipitated proteins which were subsequently investigated in heterogeneous mixtures with various reagents. The method of Todrick and Walker consisted in heating the protein suspension with varying quantities of the oxidation-reduction dye, 2,6-
dichlorophenol indophenol. This dye reacts slowly with protein sulfhydryl at ordinary temperatures. Its reaction with cysteine is not stoichiometric. Kuhn and Desnuelle have introduced the use of porphyrindin for the estimation of protein sulfhydryl groups (15). This dye is a powerful oxidizing agent, reacts rapidly and stoichiometrically with cysteine and with protein—SH groups in the cold, and possesses an $E'_0 = +0.57$ volt at pH 7.0 in comparison with an $E'_0$ for the indophenol dye of $+0.22$ volt, at the same pH. The porphyrindin dye is a deep blue in the oxidized state and slightly yellow in the reduced condition. Such a molecule possesses considerable advantages in the present connection and opens many possibilities in other fields as an intermediate of high potential.

It was thought of interest to extend the use of this dye to the estimation of sulfhydryl groups of native and denatured proteins. A remarkable method of altering many proteins is to dissolve them in urea or other amide solutions, whereby, in certain proteins, as Hopkins first pointed out, free—SH groups make their appearance (12). No estimation of the number of—SH groups liberated by this reagent has yet been made. In all proteins investigated, with the possible exception of egg albumin, the appearance of—SH groups in urea solution is coincident with dissociation of the protein molecule (6, 7). Steinhardt (30) has further shown that the functional properties of hemoglobin and pepsin are retained in urea solutions. This is a result of some significance, inasmuch as the usual concept of denaturation, implying a loss of specific properties, cannot be applied to solutions of protein in urea in spite of the apparent deep seated changes occurring in the molecule.

The study of proteins in urea solutions possesses the advantages of working in a homogeneous medium. With the porphyrindin dye, the quantitative estimation of free—SH groups at room temperature is a rapid and accurate procedure. It was thought of interest to begin this series of studies with crystalline egg albumin in urea solutions. In an attempt to find types of dispersive agents other than amides which would have the same effect as urea on the protein, the discovery was made that guanidine and

---

1 Williams and Watson have claimed that egg albumin is dissociated in 50 per cent urea (36).
methylguanidine, or more properly the guanidonium ion and the methyl guanidonium ion, were by far the most powerful agents of this kind. Either ion acted in much lower concentration than urea, and at the highest equimolecular concentrations liberated approximately 20 per cent more —SH groups. In the presence of these ions about double the number of —SH groups were found than in denaturation by either heat or precipitation. The effect of several derivatives of urea and of guanidine, such as N-methylurea and its isomer O-methylisourea, as-dimethylguanidine, and the guanidine-substituted acids was investigated and yielded comparative results of much interest.

EXPERIMENTAL

The egg albumin employed was crystallized six times and dialyzed until free of sulfate. It was used in several concentrations, each concentration being determined by both nitrogen and dry weight analyses. Two different preparations of protein were used.

The porphyrindin dye was synthesized according to Piloty and Schwerin (26) and Kuhn and Franke (16) and made up in fresh solution before each run. It was standardized against cysteine before and after every series of measurements. The dye solution is quite stable for 1 hour, but its titer slowly drops after this time. The standardization was conducted by dissolving 25 mg. of cysteine hydrochloride in water, adding sufficient dilute NH₃ to bring the pH to 7.0, and making up to a volume of 50 cc. 1 cc. of this solution reacted stoichiometrically with the dye (65 mg. of porphyrindin in 100 cc. of solution), requiring 0.62 cc. of the dye. The reaction is instantaneous and the end-point quite sharp. In order to see whether this reaction is stoichiometric for more complex sulphydryl compounds, glutathione and cysteinylcysteine (9) were titrated with the dye. Table I illustrates the quantitative nature of the reactions.

Inasmuch as the titrations of the protein were conducted in urea and in guanidine solutions, the above titrations of sulphydryl compounds were repeated in the presence of these two substances.

Svedberg (32) has stated that guanidine hydrochloride has a strong effect in dissociating the molecule of Helix hemocyanin.
Exactly the same results were obtained, however, and the possibility of urea or guanidine having any effect on the dye may be ruled out.

The solutions of egg albumin, always at pH 7.0, at various concentrations were first tested with dye and gave a negative test for sulfhydryl. When treated with either urea or guanidine hydrochloride, the maximum number of –SH groups for each concentration of these reagents appeared within a half an hour at 25°, and further standing up to 3 hours showed neither an increase nor a diminution in this number of groups. The titrations were, therefore, performed at an interval of 45 minutes from the time of mixing the protein solution with the reagents. The pH of the solutions was always at 7.0 and the temperature 25°.

### TABLE I

*Reaction of Porphyrindin with –SH Compounds*

<table>
<thead>
<tr>
<th>Substance (1.0 cc.)</th>
<th>Dye calculated</th>
<th>Dye found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.62</td>
<td>0.60</td>
</tr>
<tr>
<td>Cysteinylcysteine</td>
<td>1.24</td>
<td>1.22</td>
</tr>
</tbody>
</table>

The titrations for each concentration were performed in quadruplicate and were performed again the following day. In all cases the end-points were checked by the nitroprusside reaction. The readings among the quadruplicates at each point checked among themselves to within a drop of the dye solution. Under the conditions employed, the dye is rapidly decolorized by the protein —SH groups and each reading requires no more than a minute or two. As a further partial check, the color developed by the protein solutions at several points with nitroprusside was compared with equivalent amounts of cysteine. The agreement in all cases was excellent.3

3 It is hardly probable that the dye would react with other types of reducing groups in the protein. Such groups, involving tyrosine and tryptophane radicals, as Mirsky and Anson (22) point out, only begin to...
The first experiments to be attempted were concerned with the effect of varying the amounts of urea in solutions of protein at two different concentrations. Table II illustrates the results obtained. It is apparent that the relative percentage of \(-\text{SH}\) groups liberated by a definite amount of urea is not affected by the protein concentration, although the relative volumes of the solutions studied were quite different. The results are always finally expressed in terms of cysteine concentration, or cysteine per hundred gm. of protein.

### Table II

**Effect of Various Urea Concentrations**

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Urea added</th>
<th>Dye</th>
<th>Cysteine per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>per cent</strong></td>
<td>gm.</td>
<td>cc.</td>
<td></td>
</tr>
<tr>
<td>7.7 (Protein solution 1.0 cc.)</td>
<td>1.0</td>
<td>1.35</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>1.20</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>1.15</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>0.81</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.37</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.85 (Protein solution 2.0 cc.)</td>
<td>2.0</td>
<td>1.35</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>1.20</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>1.16</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>0.85</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>0.38</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

On a molar basis, 1 gm. of urea is equivalent to 1.6 gm. of guanidine hydrochloride. The effect of the latter substance on a solution of egg albumin is described in Table III. It is observed that the effect is strikingly different from that of urea. First of all, it is apparently stronger, more \(-\text{SH}\) groups are liberated, and, secondly, the effect begins at much lower concentrations of guanidine. Moreover, the maximum effect extends over a wide range of guanidine concentration.

make their presence felt at pH 10 and, moreover, react very slowly with ferricyanide and not at all with cystine or phosphotungstate. It is certain in any case that they would not exhibit a nitroprusside reaction.
The effect of liberating —SH groups just begins with urea when 0.5 gm. is added to 1 cc. of the protein solution. With guanidine hydrochloride, this effect becomes evident when the concentration is equivalent to 0.25 gm. of urea per cc. of protein solution. At the lowest effective concentrations, guanidine is thus twice as potent as urea.

It was further observed that following oxidation by the dye, the higher concentrations of protein often set to a gel. In the presence of urea, these gels were clear and translucent; those in the presence of guanidine were invariably milky and turbid.

A further series of experiments was set up to determine more closely the relation of protein concentration to the amount of sulfhydryl liberated. Table IV presents the results over a fairly wide range of protein concentration. In all, the urea concentration was invariably 1 gm. per cc. of protein solution, the guanidine hydrochloride 1.6 gm. per cc. of protein solution, and thus equimolar amounts.

The results may be consistently interpreted by the fact that, at least within the range of concentrations investigated, the proportion of —SH groups liberated by either urea or guanidine is independent of the protein concentration. Comparison of the results with urea and guanidine at equimolar concentrations shows that the effect induced by the latter is consistently greater than that by the former. The difference between the average values for each substance amounts to nearly 20 per cent.

An attempt was made to estimate the —SH groups liberated by the heat denaturation of egg albumin at pH 7.0. 2 cc. of 3.78 per cent solution of the protein were set in a boiling water bath for half an hour, then quickly cooled, and titrated with the dye. There was found 0.50 per cent of cysteine, which may be compared with the result of Todrick and Walker of 0.63 per cent (34) and the results of Mirsky and Anson (21, 22) ranging from 0.55 to 0.62 per cent. Kuhn and Desnuelle found 0.58 per cent for this protein (15).

It was thought of interest to observe the magnitude of the effect evoked by various derivatives of urea and of guanidine.

When the protein solution is heated in the presence of urea or guanidine, the same number of —SH groups is liberated as in the unheated protein treated with these substances.
TABLE III

Effect of Guanidine on Egg Albumin

Protein concentration, 3.85 per cent; protein solution, 2.0 cc. in each case.

<table>
<thead>
<tr>
<th>Guanidine hydrochloride added (gm.)</th>
<th>Dye (cc.)</th>
<th>Cysteine (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.20</td>
<td>1.80</td>
<td>1.28</td>
</tr>
<tr>
<td>2.20</td>
<td>1.80</td>
<td>1.28</td>
</tr>
<tr>
<td>1.60</td>
<td>1.80</td>
<td>1.28</td>
</tr>
<tr>
<td>1.20</td>
<td>1.80</td>
<td>1.28</td>
</tr>
<tr>
<td>1.00</td>
<td>1.80</td>
<td>1.28</td>
</tr>
<tr>
<td>0.80</td>
<td>1.80</td>
<td>1.28</td>
</tr>
<tr>
<td>0.60</td>
<td>Gel</td>
<td>0.18</td>
</tr>
<tr>
<td>0.40</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE IV

Relation of Protein Concentration to -SH Produced

<table>
<thead>
<tr>
<th>Protein concentration (per cent)</th>
<th>Protein solution (cc.)</th>
<th>Urea (cc.)</th>
<th>Guanidine (cc.)</th>
<th>Cysteine (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>1.0</td>
<td>1.35</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>5.45</td>
<td>2.0</td>
<td>2.15</td>
<td>2.35</td>
<td>1.10</td>
</tr>
<tr>
<td>4.36</td>
<td>2.0</td>
<td>1.54</td>
<td>1.91</td>
<td>0.98</td>
</tr>
<tr>
<td>3.85</td>
<td>2.0</td>
<td>1.35</td>
<td>1.80</td>
<td>0.97</td>
</tr>
<tr>
<td>3.78</td>
<td>2.0</td>
<td>1.38</td>
<td>1.65</td>
<td>0.99</td>
</tr>
<tr>
<td>2.72</td>
<td>2.0</td>
<td>1.00</td>
<td>1.35</td>
<td>1.00</td>
</tr>
<tr>
<td>1.09</td>
<td>2.0</td>
<td>0.39</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>1.24</td>
</tr>
</tbody>
</table>

TABLE V

Effect of Derivatives of Urea and of Guanidine

Protein solution (5.45 per cent) 1.0 cc. in each case.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Weight of substance (gm.)</th>
<th>Dye (cc.)</th>
<th>Cysteine (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>1.0</td>
<td>1.05</td>
<td>1.06</td>
</tr>
<tr>
<td>N-Methylurea</td>
<td>1.23</td>
<td>1.02</td>
<td>1.02</td>
</tr>
<tr>
<td>O-Methylisourea hydrochloride</td>
<td>1.84</td>
<td>1.04</td>
<td>1.05</td>
</tr>
<tr>
<td>Guanidine hydrochloride</td>
<td>1.60</td>
<td>1.19</td>
<td>1.20</td>
</tr>
<tr>
<td>Methylguanidine hydrochloride</td>
<td>1.80</td>
<td>1.18</td>
<td>1.19</td>
</tr>
<tr>
<td>as-Dimethylguanidine hydrochloride</td>
<td>2.00</td>
<td>0.75</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Table V lists the results obtained with equimolecular concentrations of urea, N-methylurea, and the hydrochlorides of O-methylisourea, guanidine, methylguanidine, and \textit{as}-dimethylguanidine. The effect of urea is similar to that of its derivatives, guanidine and methylguanidine possess an apparently equal effect, whereas asymmetric dimethylguanidine is relatively weaker.

Inasmuch as the protein solution had in all cases been brought to pH 7.0 with dilute NH$_4$H$_2$, there was necessarily always NH$_4$Cl present in solutions containing the hydrochlorides of the molecules. Svedberg (32) has shown that NH$_4$Cl, but not other chloride salts, augments the dissociation of certain proteins by weakly effective agents. In order to eliminate the possibility of this salt entering into the action of the hydrochlorides described in Table V, the experiments were repeated in protein solutions which had been brought to neutral pH with dilute NaOH. In all cases the results were identical with those in Table V. Furthermore, ammonium chloride was added to protein solutions containing the minimum amounts of urea, N-methylurea, and guanidine hydrochloride which were effective in producing sulfhydryl groups in the protein. In no case was any increase in these groups apparent.

Finally, it may be mentioned that the following substances produced no sulfhydryl groups in protein solutions: arginine, N-acetylariginine, homoarginine, anhydroluguanidocystine, glycocyamine, creatine, and creatinine.

**DISCUSSION**

Although the effect produced on the protein by solutions of urea, guanidine, and their derivatives is very striking, a simple explanation of such effect appears rather difficult at present. Any attempt to interpret the differences in the magnitude of the effects produced by the various substances must take into account the various forms in which each may occur in solution. Urea and N-methylurea exist as electrically neutral molecules; O-methylisourea, guanidine, methylguanidine, and \textit{as}-dimethylguanidine exist as positively charged ions. All of these substances, however, possess one property in common, the capacity of existing in several resonating forms in solution. Urea, for example, may be represented as resonating among the following three structures.
The thermochemical behavior of the amides (25), their high dipole moment values (17), and their influence on acidic dissociation (38) are best interpreted by the effect of such resonating forms. Replacement of hydrogen by methyl, as in N-methylurea, might conceivably tend to hinder the double bond from migrating to the methylated nitrogen, owing to the greater electronegativity of carbon over hydrogen. With asymmetric N,N-dialkyl substitution this hindrance might be expected to be somewhat greater. A very much greater effect would be expected for symmetrical N,N'-dialkyl substitution, for here the resonance of the double bond would be considerably restricted, and the neutral form

\[
\begin{align*}
\text{O} & \quad \text{C} \\
\text{NHCH}_3 & \quad \text{NHCH}_3
\end{align*}
\]

would be much more important than the other two charged forms. N-Methylurea, as well as its isomer, O-methylisourea, has the same effect as urea on the protein (Table V), whereas symmetrical N,N'-diethylurea according to Hopkins (12) has no effect whatever.

Guanidine in the form of the guanidonium ion, like urea, resonates among the following three structures (25).

\[
\begin{align*}
\text{NH}_2 & \quad \text{NH}_2^+ \\
\text{C} & \quad \text{C} \\
+\text{NH}_2 & \quad \text{NH}_2
\end{align*}
\]

Substitution of a methyl group for hydrogen in this ion, as in the analogous case of urea, leads to the same effect on the protein as the parent substance produced (Table V). On the other hand, the \textit{as}-dimethylguanidonium ion, in which resonance may be expected to be more restricted, actually shows a distinctly smaller
Egg albumin possesses a total sulfur percentage of 1.62 according to Osborne (23) and 1.60 according to Baernstein (3). The former investigator also estimated the alkali-labile sulfur, presumably cystine and cysteine sulfur, to amount to 0.49 per cent; Zahnd and Clarke found 0.44 per cent (37). The percentage of cysteine based on the labile sulfur data would then be respectively 1.84 and 1.66 for this protein. No methods have hitherto been devised to determine what proportion of the labile sulfur in the native protein is cysteine or cystine. In any case, treatment with urea exposes some 60 per cent, treatment with guanidine 67 to 73 or approximately 70 per cent of the total alkali-labile sulfur content. However, part of the labile sulfur undoubtedly must belong to cystine, but the results of this paper show that only a relatively small amount of this amino acid must exist in egg albumin. By far the greater amount, up to about 70 per cent of the labile sulfur, must belong to cysteine.

From the latest value of 40,000 for the molecular weight of egg albumin (18), it would appear that there are 6 atoms of alkali-labile sulfur per molecule of this protein. On the basis of approximately 70 per cent of this type of sulfur being due to cysteine, it would appear that 4 of the 6 atoms of sulfur, or 67 per cent, were due to cysteine. This would imply that in a molecule of egg albumin, there existed at least 4 molecules of cysteine, which are revealed in solutions of guanidine. Since heat or precipitation denaturation liberates about half as much cysteine as does guanidine, it would appear that 2 molecules of cysteine are revealed by the former processes.

The manner in which the sulfhydryl groups of the native proteins are masked presents a unique problem. Astbury and Dickinson (2) have shown that the globular proteins when de-
natured exist in the β-keratin or stretched type of configuration. In the native state, such proteins may be assumed to exist in a coiled or contracted configuration. Several forces, notably those existing between polar groups on the side chains, can be considered as supplying the binding agents for the contracted state of the native protein. Among such polar groups, the —SH group must, at least for egg albumin, take an important place. With what type of grouping it may be linked in the native protein, in such a way that it is chemically inert, is not at present clear. The investigations of Schubert (27) on the semimercaptal linkages are suggestive in this respect. In any case, no matter in what order of events the phenomenon occurs, the action of urea or guanidine probably results in a transition of the contracted to the extended configuration of the protein, the original binding forces, including —SH groups, are dissipated, and the latter may then be chemically estimated. The effect of dissipating these binding forces is evidently very much greater through guanidine than through urea. The problem, therefore, which presents itself most insistently is to find that type of linkage involving mercaptides which is dissolved by urea or guanidine.6

**SUMMARY**

1. The use of the high potential dye, porphyrindin, for the estimation of sulfhydryl groups in proteins has been extended to the study of egg albumin in solutions of urea, guanidine, and their derivatives at pH 7.0.

2. Egg albumin in solutions of 16.6 mM of urea added per cc. of protein solution showed a cysteine content amounting to 1.00 per cent. In equimolar solutions of guanidine hydrochloride, the cysteine content amounts to 1.24 per cent. These values

---

6 Some authors believe that the process of denaturation liberates sulfhydril groups by the hydrolysis of the S—S linkage of cystine. Speakman has interpreted the behavior of wool fibers in steam on this basis (29). Whatever may be the behavior of keratins on heating, it is doubtful whether urea and guanidine act on protein S—S linkages in such a way as to form —SH groups. No such reaction is known for simpler dithio compounds and attempts to find —SH groups on treatment of a number of cystine derivatives have proved fruitless. There is no effect whatever of urea or guanidine on substances such as cystinylcystine, anhydrocystinylcystine, cystinylbiglycine, or cystinylidiglycine.
512 Sulfhydryl Groups in Proteins. I

are about double those reported for heated or precipitated egg albumin. Not only does guanidine possess a stronger effect in liberating sulfhydryl groups than urea, but it shows the maximum capacity at relatively lower concentrations. The percentage of sulfhydryl groups demonstrated in these solutions is independent of the protein concentration and depends only on the concentrations of urea or of guanidine.

3. The cysteine calculated from the present results amounts at the most in urea to some 60 per cent, in guanidine to about 70 per cent of the total alkali-labile sulfur of egg albumin. This would indicate that the greater amount of the alkali-labile sulfur belonged to cysteine, a much smaller amount to cystine. Of the 6 atoms of alkali-labile sulfur in the molecule of this protein, 4, or 67 per cent, apparently belong to cysteine.

4. The effects of urea, guanidine, and their derivatives are briefly discussed in terms of the resonance of these molecules. In cases like sym-diethylurea or as-dimethylguanidine, where resonance may be expected to be considerably restricted, little or no effect on the protein seems to be produced.

5. Neither urea nor guanidine has any effect on various complex peptides of cystine. It is suggested that the effect of the former substances is not on disulfide bonds, but is concerned with a dissolution of linkages within the intact protein which involve the sulfhydryl groups of cysteine. It would appear probable that such linkages were at least in part responsible for the native state of the protein.

BIBLIOGRAPHY

SULFHYDRYL GROUPS IN PROTEINS: I. EGG ALBUMIN IN SOLUTIONS OF UREA, GUANIDINE, AND THEIR DERIVATIVES

Jesse P. Greenstein


Access the most updated version of this article at http://www.jbc.org/content/125/2/501.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/125/2/501.citation.full.html#ref-list-1