Egg albumin in the native, unaltered state, does not give tests characteristic of sulfhydryl groups. When, however, this protein is treated in any one of several ways, such as by heat (8, 9, 17), ultraviolet irradiation (10), shaking (10), or by solution in urea or other amides (7), free sulfhydryl groups make their appearance. The amount of free –SH groups appearing in egg albumin through the action of urea, guanidine hydrochloride, and their derivatives has been estimated (6). Considerable differences in the number of such groups liberated, which depend on the nature and the concentration of the reagent used, were noted.

The investigation of these groups has been extended to a number of proteins whose molecular weights have been reported to be smaller in urea solutions than in water. The three seed globulins, edestin, excelsin, and amandin, have practically the same molecular weight in water (4, 5, 15, 16). Their molecular weights in 4 M urea are also almost the same (4), but are reported to be only one-eighth as great as in water. Burk and Greenberg (5) showed that the molecular weight of horse hemoglobin in urea solution was 34,000, a value just half the molecular weight of this protein in water. The results of these investigators were confirmed by Wu and Yang (18) and by Steinhardt (14), the latter showing further that the molecular weight in acetamide solutions was comparable to that in urea.

It has been suggested that the difference in the molecular weights of certain proteins in water and in urea is due in part to a scission of the S—S linkages of the protein in the latter solvent with the
subsequent appearance of —SH groups (4). It seemed desirable, therefore, to study these groups in proteins dissolved in this and related solvents.

EXPERIMENTAL

Preparation of Proteins—Edestin was prepared from ground hemp seed by the method of Svedberg and Sjögren (16). Five different preparations were employed. The protein was dissolved in several concentrations in 10 per cent sodium chloride solution. The concentrations of protein were estimated by nitrogen analyses, assuming Bailey's value of 18.4 for edestin nitrogen (3). Excelsin was prepared from Brazil nuts by a slight modification of the method of Burk (4). Three different preparations were used. Like edestin, the protein was dissolved in varying concentrations in 10 per cent sodium chloride. The concentrations of protein were estimated, assuming Osborne's value of 18.3 per cent nitrogen (12). Amandin was prepared from ground almonds according to the method of Burk (4) and dissolved in 10 per cent sodium chloride. Osborne's value of 19.3 per cent nitrogen for this protein was used to estimate the concentration (12).

Globin was prepared from denatured horse globin by the method of Anson and Mirsky (1). The solution of globin, during the dialysis from ammonium sulfate, deposited a copious precipitate of denatured material. The filtered solution, containing native globin, amounted to 3.70 per cent in concentration from both nitrogen analyses (16.9 per cent for globin N) and dry weight determinations. Studies were made on fresh solutions, because the denaturation of this protein, under most conditions, is fairly rapid.

Estimation of —SH Groups—A convenient method for the estimation of protein —SH groups consists in titrating the protein with porphyrindin, a method first introduced by Kuhn and Desnuelle (8). This dye is a powerful oxidizing agent and reacts rapidly and

1 It has been shown that none of the reagents such as urea and guanidine hydrochloride, which act on certain proteins in such a way that —SH groups appear, has any effect on the following complex peptides of cystine: cystinylcystine, bisanhydrocystinylcystine, cystinyliddiglycine, and cystinylidiglycine (6).

2 Wu and Yang (18) have shown that the globins of various species have the same properties in urea as the parent hemoglobins.
stoichiometrically with sulphhydryl groups at a neutral reaction and
at room temperature. When the dye is added to an aqueous
solution of egg albumin (6), edestin, excelsin, amandin, or globin,
no apparent reduction occurs. When, however, these proteins,
with the exception of amandin, are dissolved in solutions of urea,
guanidine hydrochloride, and some of their derivatives, free —SH
groups appear, and a certain amount of the dye added to such
solutions becomes reduced to the colorless leuco form (6). For
convenience, the amount of dye reduced by the protein is expressed
in terms of its equivalent of cysteine per 100 gm. of protein (6, 8).

The titrations were conducted by adding to the solution of
protein in urea, guanidine hydrochloride, and to their derivatives, a
solution of the porphyrindin dye until a negative nitroprusside
reaction was obtained. All determinations were performed in
sextuplicate. The temperature was 30°. Before and after
every series of measurements, the dye was titrated against a
standard cysteine solution (6). For the studies with the seed
globulins in 10 per cent sodium chloride solution the dye was
used at a lower concentration than in the studies with globin,
which were conducted in water. The lower concentration of
dye in the former case was employed partly because of its
lower solubility in salt solution, and partly because of the
relatively small number of sulphhydryl groups which appeared in
these proteins.

The first experiments were concerned with studying the effect
of urea and guanidine hydrochloride in equimolar amounts on
different concentrations of the proteins. No effect whatever of
these reagents was found with amandin. Neither the preparation
of amandin made in this laboratory, nor one which had been made
by T. B. Osborne, gave a sulphhydryl reaction in either urea or
guanidine hydrochloride. The fact that no reduction of the
porphyrindin dye occurred with this protein in solutions of urea
and guanidine hydrochloride indicated that the dye was unaffected

3 The presence of free sulphhydryl groups in the protein can be followed by
a positive nitroprusside reaction. After the addition of a sufficient amount
of dye to the protein, this reaction becomes negative.

4 I am greatly indebted to Dr. Vickery for an Osborne preparation of
amandin and to Dr. Hitchcock for a sample of edestin.
by any groups other than sulfhydryl (11), which may have been liberated by the reagents. The results on edestin, excelsin, amandin, and globin are reported in Table I, expressed as cysteine per 100 gm. of protein.

Edestin, excelsin, and globin were further investigated in solutions of varying concentration of guanidine hydrochloride. The

<table>
<thead>
<tr>
<th>Protein</th>
<th>Per cent protein in</th>
<th>Dye</th>
<th>Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>concentration</td>
<td>Urea</td>
<td>Guanidine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m M X 10^-4</td>
<td>HCl</td>
</tr>
<tr>
<td>Edestin</td>
<td>0.98</td>
<td>2.66</td>
<td>4.17</td>
</tr>
<tr>
<td></td>
<td>1.47</td>
<td>4.17</td>
<td>6.42</td>
</tr>
<tr>
<td></td>
<td>1.69</td>
<td>4.75</td>
<td>7.29</td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td>5.33</td>
<td>8.34</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>6.95</td>
<td>10.42</td>
</tr>
<tr>
<td>Excelsin</td>
<td>5.30</td>
<td>3.59</td>
<td>7.54</td>
</tr>
<tr>
<td></td>
<td>8.20</td>
<td>5.00</td>
<td>12.74</td>
</tr>
<tr>
<td></td>
<td>10.90</td>
<td>7.07</td>
<td>14.48</td>
</tr>
<tr>
<td></td>
<td>11.00</td>
<td>6.40</td>
<td>15.18</td>
</tr>
<tr>
<td>Amandin</td>
<td>10.50</td>
<td>&lt;0.07</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td></td>
<td>1.85</td>
<td>2.71</td>
<td>8.13</td>
</tr>
<tr>
<td></td>
<td>2.77</td>
<td>4.06</td>
<td>12.18</td>
</tr>
<tr>
<td></td>
<td>3.70</td>
<td>5.42</td>
<td>16.26</td>
</tr>
</tbody>
</table>

very strong effect of this reagent is revealed in Table II, the results being comparable to those obtained on egg albumin (6).

In Table III is described the effect of various derivatives of urea and guanidine hydrochloride on the proteins. For comparison, the data on egg albumin (6) are included. All the reagents were used in equimolar concentrations.

6 In the dried, solid state, the dye appears to be quite stable. Material which had stood for several months at room temperature showed no loss in titer in fresh solution.
### TABLE II

*Effect of Varying Concentrations of Guanidine Hydrochloride on Edestin, Excelsin, and Globin*

Edestin was employed in 1.96 per cent solution, excelsin in 8.20 per cent, and globin in 3.70 per cent. In each test 2.0 cc. of protein solution were used. In edestin and excelsin solutions the dye was used in $1.16 \times 10^{-2}$ M concentration (0.0325 per cent) in 10 per cent NaCl; in the globin solutions the dye was used in $2.32 \times 10^{-3}$ M concentration (0.065 per cent) in water.

<table>
<thead>
<tr>
<th>Guanidine HCl added</th>
<th>Edestin</th>
<th>Excelsin</th>
<th>Globin</th>
</tr>
</thead>
<tbody>
<tr>
<td>gm.</td>
<td>Dye $mM \times 10^{-4}$</td>
<td>Cysteine per cent</td>
<td>Dye $mM \times 10^{-4}$</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.4</td>
<td>2.32</td>
<td>0.14</td>
<td>3.48</td>
</tr>
<tr>
<td>0.8</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
<tr>
<td>1.2</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
<tr>
<td>1.6</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
<tr>
<td>2.0</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
<tr>
<td>2.4</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
<tr>
<td>2.8</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
<tr>
<td>3.2</td>
<td>8.11</td>
<td>0.50</td>
<td>12.76</td>
</tr>
</tbody>
</table>

### TABLE III

*Effect of Urea, Guanidine Hydrochloride, and Their Derivatives on Proteins*

The concentration of the protein solutions was as follows: egg albumin 5.45 per cent, edestin 2.45 per cent, excelsin 8.2 per cent, and globin 3.70 per cent.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount of reagent added per cc protein solution</th>
<th>Egg albumin</th>
<th>Edestin</th>
<th>Excelsin</th>
<th>Globin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>gm.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>0.34</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>N-Methylurea</td>
<td>1.23</td>
<td>1.02</td>
<td>$&lt;0.03$</td>
<td>$&lt;0.01$</td>
<td>$&lt;0.03$</td>
</tr>
<tr>
<td>O-Methylisourea hydrochloride</td>
<td>1.84</td>
<td>1.05</td>
<td>0.17</td>
<td>$&lt;0.01$</td>
<td>$&lt;0.03$</td>
</tr>
<tr>
<td>Guanidine hydrochloride</td>
<td>1.60</td>
<td>1.20</td>
<td>0.51</td>
<td>0.18</td>
<td>0.56</td>
</tr>
<tr>
<td>Methylguanidine hydrochloride</td>
<td>1.82</td>
<td>1.19</td>
<td>0.16</td>
<td>0.07</td>
<td>0.56</td>
</tr>
<tr>
<td>$a$-Dimethylguanidine hydrochloride</td>
<td>2.00</td>
<td>0.75</td>
<td>$&lt;0.03$</td>
<td>$&lt;0.01$</td>
<td>$&lt;0.03$</td>
</tr>
<tr>
<td>Acetamide</td>
<td>1.0</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.03$</td>
<td>$&lt;0.01$</td>
<td>$&lt;0.03$</td>
</tr>
</tbody>
</table>
DISCUSSION

As in egg albumin, the proportion of sulfhydryl groups liberated in edestin, excelsin, and globin is apparently independent of the protein concentration (Table I). The behavior of edestin, excelsin, and globin toward varying concentrations of guanidine hydrochloride (Table II) is also strikingly similar to that of egg albumin (6) in that guanidine hydrochloride reaches its maximum effect at a comparatively low concentration.

The comparison of the effects of various derivatives of urea and of guanidine hydrochloride in Table III reveals not only the uniformly stronger action of guanidine hydrochloride over urea for all of the proteins studied, but also the relatively weak action of the methylated ureas on edestin, excelsin, and globin. In egg albumin, on the other hand, the methylated ureas are as effective as the parent substance. Methylguanidine hydrochloride exerts a weak effect on edestin and excelsin, but in globin, as in egg albumin, its action is equal to that of guanidine hydrochloride itself. Dimethylguanidine hydrochloride and acetamide have little or no effect on edestin, excelsin, or globin.

Osborne's early investigations on the analytical data for edestin and excelsin led to a value for the molecular weight of the former of 14,523 and for the latter of 14,738, or some multiple of these (12). Osborne's value (12) as well as that of Zahnd and Clarke (19) for the alkali-labile sulfur in edestin is 0.35 per cent. The alkali-labile sulfur in excelsin is also 0.35 per cent (12). These results lead to the assumption of 3 atoms of alkali-labile sulfur in each protein of molecular weight between 28,000 and 29,000.

When edestin is dissolved in solutions of guanidine hydrochloride, 0.51 per cent cysteine appears (Table III). On the basis of a molecular weight of 28,000, this amounts to 1 mole of cysteine per mole of protein. In excelsin dissolved in guanidine hydrochloride, the cysteine which appears amounts to 0.17 per cent. On the basis of a molecular weight of 28,000, this amounts to 0.3 mole of cysteine per mole of protein, or better, 1 mole of cysteine per mole of protein of molecular weight 84,000.

Amandin, according to Osborne, has 0.22 per cent of alkali-labile sulfur (12). On the basis of a molecular weight of 28,000 to 29,000 (12), there would appear to be 2 atoms of alkali-labile sulfur per mole of protein. It is probable that all the alkali-labile sulfur of this protein belongs to cystine.
conditions, therefore, the cysteine which appears in guanidine hydrochloride solutions of edestin amounts to 3 times that which appears in excelsin.

Schulz reported for horse globin a value of 0.20 per cent for the alkali-labile sulfur (13). On the basis of a molecular weight for globin of approximately 66,000, there would appear to be 4 atoms of alkali-labile sulfur per mole of protein. When this protein is dissolved in guanidine hydrochloride solutions, there appears 0.56 per cent cysteine, or 3 moles of cysteine per mole of protein.\(^7\)

To summarize the results obtained thus far on the proteins in guanidine hydrochloride solutions: in equal concentrations of this reagent, the cysteine determined amounts in egg albumin to two-thirds, in edestin to one-third, in excelsin to one-ninth, and in globin to three-fourths of the alkali-labile sulfur.

Although the molecular weights in urea of edestin, excelsin, and amandin appear to be very nearly the same (4), the proportion of cysteine which appears in the three proteins is very different (Table I). Hemoglobin has the same molecular weight in either urea or in acetamide, namely half that found in water (14); in globin, however, cysteine appears in urea, not in acetamide. It seems, therefore, that no obvious relation exists between the differences of molecular weight in different solvents and the appearance of sulfhydryl groups. The latter in all probability exist as part of the cysteine residues within the native protein, and do not owe their origin to scission of disulfide linkages.

It is evident that the present results may have broad implications for the problem of protein denaturation. The number of sulfhydryl groups liberated, which may be taken as a measure of the change in protein configuration, is clearly different according to the nature and the concentration of the individual denaturing agent. Further studies of the effect of urea, guanidine hydrochloride, and their derivatives on other properties of the proteins are at present under way.

**SUMMARY**

1. Edestin, excelsin, amandin, and globin in aqueous solution do not give tests characteristic of sulfhydryl groups. When these

\(^7\) Anson and Mirsky (2) found the cysteine content of acid-acetone globin to be 0.38 per cent. This amount of cysteine is equivalent to 2 moles per mole of protein.
proteins, with the exception of amandin, are treated with urea, guanidine hydrochloride, and certain of their derivatives, free sulfhydryl groups appear. The estimation of these groups is accomplished by the use of the porphyrindin dye. The results are expressed in terms of cysteine.

2. The proportion of cysteine which appears is independent of the protein concentration. Guanidine hydrochloride exerts the strongest action of all the reagents studied, and reaches its maximum effect at a comparatively low concentration. The methylated ureas as well as methylguanidine hydrochloride have a weak effect on edestin and excelsin. In globin, methylguanidine hydrochloride is as effective as the parent substance. Dimethylguanidine hydrochloride and acetamide have little or no effect on edestin, excelsin, and globin.

3. The relation of the cysteine which appears in solutions of guanidine hydrochloride to the alkali-labile sulfur of the proteins has been considered.

4. There does not appear to be any correlation between the differences in molecular weight of these proteins in water and in solutions of certain amides and the amount of cysteine which may appear in the latter solvents.

BIBLIOGRAPHY

SULFHYDRYL GROUPS IN PROTEINS: II. EDESTIN, EXCELSIN, AND GLOBIN IN SOLUTIONS OF GUANIDINE HYDROCHLORIDE, UREA, AND THEIR DERIVATIVES
Jesse P. Greenstein


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