The Solubility of Amino Acids and Related Compounds in Aqueous Urea Solutions*

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(Received for publication, July 15, 1963)

The traditional explanation for the denaturation of globular proteins by urea has been that urea, because of its structural similarity to a peptide group, is able to form stronger hydrogen bonds with peptide groups than water does, and that it is therefore able to “break” interpeptide hydrogen bonds which water cannot break. A number of recent investigations (1-6) have thrown doubt upon this explanation, for two reasons. (a) Interpeptide and other hydrogen bonds have been shown to be relatively unimportant as a source of free energy stabilizing the native globular proteins in water solution. The major force stabilizing the globular structure is believed instead to be the hydrophobic force which arises from the unfavorable interaction between nonpolar parts of a protein molecule and water (7, 8). Thus a good denaturing agent might be expected to be one which weakens hydrophobic forces rather than interpeptide and other hydrogen bonds. That urea might be able to play this role was suggested some time ago by Waugh (9), and experimental evidence for it has come from the finding of Bruning and Holtzer (10) that urea decreases the stability of detergent micelles in water. (b) The basic assumption that urea forms stronger hydrogen bonds than water does has been shown to be highly questionable, especially by studies of Klotz and Franzen (11) which purport to show that hydrogen bonds between water and the peptide group of N-methylacetamide are considerably stronger than interpeptide bonds between the amide molecules.

A reinvestigation of the denaturing action of urea thus seems appropriate. A direct approach to the problem is to study the effect of urea on the solubility of a variety of compounds in aqueous solution, and it is especially appropriate to study the solubility of amino acids and of peptides, as these molecules contain the same reactive groups as proteins do. The present investigation was therefore initiated about 2 years ago. Preliminary results (12) showed at once that aqueous urea solutions are indeed able to accommodate nonpolar amino acid side chains better than water can. (The same effect was demonstrated independently for hydrocarbons themselves by Wetlauffer (13).) Our preliminary paper showed also, however, that urea stabilizes the polar side chain of asparagine, from which one may infer that urea also interacts favorably (i.e. with a decrease in free energy) with peptide groups. That it actually does so has recently been asserted forcefully by Robinson and Jencks (14), who have studied the solubilities of two small peptides in aqueous urea and in other solvents.

The solubilities of 11 amino acids, two peptides, and two carboxybenzoxyl derivatives reported in this paper confirm that urea possesses both of these properties; i.e. it stabilizes both nonpolar groups and peptide groups. That the two effects in combination account quantitatively for the denaturing action of urea will be shown in a subsequent paper.

EXPERIMENTAL PROCEDURE

The amino acids and peptides used in this study were purchased from Mann Research Laboratories. The two carboxybenzoxyl derivatives were purchased from Cyclo Chemical Corporation. For the optically active amino acids, the L isomers were used.

Since solubility curves are themselves criteria of purity (see below), these products were used without purification unless the solubility curves indicated the presence of impurity. By this criterion additional purification was required only for diglycine, triglycine, and histidine. Diglycine and triglycine were twice recrystallized from water and once from 50% ethanol. Pure histidine was prepared by neutralizing histidine hydrochloride monohydrate with a slight excess of LiOH, followed by precipitation with ethanol. The free base was twice recrystallized from water.

Grade A urea, purchased from the California Corporation for Biochemical Research, was found to possess no titratable impurity and was therefore used without further purification. When the urea was dissolved in water, a slow formation of a precipitate was observed, so that urea solutions were always freshly prepared and all measurements were completed as rapidly as possible.

All water employed in these measurements was glass-distilled and stored in a CO₂-free atmosphere.

Solubility Measurements—Weighed amounts of sample and solvent were placed into glass tubes fitted with ground glass stoppers. The stoppers were greased or fitted with Teflon sleeves to prevent leakage. (For relatively insoluble samples, 250-ml Erlenmeyer flasks were used instead of the glass tubes.) The space above the mixture was flushed with nitrogen before the tubes or flasks were sealed. The vessels were completely immersed in a water bath maintained at 25.1 ± 0.01⁰C, and shaken for 24 hours or longer. The contents were filtered by suction through sintered glass disks while still in the constant temperature bath. Usually at least five mixtures were treated in this way for each sample, the solute content being varied in such a way for each sample, the solute content being varied...
way that the solute would be completely dissolved in two of the
samples and be present in varying degrees of excess in the other
three.

Several methods were used to analyze the solute content of the
filtered solutions. In pure water a dry weight procedure was
used, except for tyrosine and the two carbobenzoxy derivatives.
Duplicate aliquots were evaporated in a vacuum desiccator at 35°
and then in a forced air circulating oven at 107°. Identical dry
weights were obtained at the two temperatures, except for
asparagine and phenylalanine, both of which appeared to exist
as hydrates when dried at 35°. Since we could find no previous
mention of the existence of a stable phenylalanine hydrate, we
checked the results for phenylalanine by titration, by means of a
procedure similar to that described below for analysis in urea
solutions.

Solubilities in urea solution, except for tyrosine and the two
carbobenzoxy derivatives, were determined by titration with 0.5
M NaOH, using a Radiometer Titigraph, model SBR 2c, together
with a Radiometer Titrator, model TTTte. Typical data are
shown in Fig. 1, and it is seen that they yield directly the number
of moles of amino acid or peptide in the saturated solution.

Concentrations of tyrosine and of the two carbobenzoxy
derivatives were determined spectrophotometrically; absorbance
at 275 mµ for tyrosine, and in the range from 256 to 266 mµ for
the carbobenzoxy derivatives, was used. The molar or specific
absorbance had to be determined independently in each solvent.
For tyrosine, for example, the molar extinction coefficient varied
from 1370 M⁻¹ cm⁻¹ in water to 1440 M⁻¹ cm⁻¹ in 8 M urea.

It should be noted that the preliminary data reported from
this laboratory earlier (12) constitute a series of measurements
entirely independent of those reported here. A turbidimetric
method was used for glycine, spectrophotometry for tyrosine
and phenylalanine, and optical rotation for the remaining amino
acids.

Typical experimental data are shown in Fig. 2. They serve
not only to determine the solubility, but also as criteria for
purity. The constancy of the parameter being used for concen-
tration measurement, regardless of the amount of excess solute
originally added, is indicative of the absence of impurities.

A second criterion for purity of our solutes, and for the general
validity of our methods of measurement, is provided by compari-
sion of our solubilities in water with previously determined
values. Data available for such comparisons are shown in
Table I.

RESULTS

The solubilities determined in this study are summarized in
Table II.

We have used these data to calculate free energies of transfer
of the solutes from water to aqueous urea solutions. To do so we
have expressed the chemical potential of a solute (designated by
subscript i) in any solvent by the relation

$$
\mu_i = \mu^o_i + RT \ln N_i + RT \ln \gamma_i
$$

(1)

where $N_i$ is the mole fraction of solute, $\mu^o_i$ is the standard chemi-
Table I
Some comparisons between solubilities in water by different methods or from different laboratories

<table>
<thead>
<tr>
<th>Soluble</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solute</td>
<td>Dry weight</td>
</tr>
<tr>
<td>Glycine</td>
<td>25.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>16.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.15</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.79</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0451$^a$</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.51</td>
</tr>
<tr>
<td>Diglycine</td>
<td>23.3</td>
</tr>
<tr>
<td>Triglycine</td>
<td>6.30</td>
</tr>
</tbody>
</table>

$^a$ Also unpublished data listed in The Handbook of Chemistry and Physics.
$^b$ Data of P. Whitney.
$^c$ Optical rotation, data of P. Whitney.
$^d$ Titrimetric, this paper.
$^e$ Spectrophotometric, this paper.

Table II
Solubilities at 25.1$^\circ$C

<table>
<thead>
<tr>
<th>Soluble</th>
<th>Solubilities at concentration of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g solvent</td>
</tr>
<tr>
<td>Glycine</td>
<td>25.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>16.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.16</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.80</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.38</td>
</tr>
<tr>
<td>Methionine</td>
<td>5.59</td>
</tr>
<tr>
<td>Threonine</td>
<td>9.80</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0451</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.30</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.30</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.51</td>
</tr>
<tr>
<td>Diglycine</td>
<td>23.3</td>
</tr>
<tr>
<td>Triglycine</td>
<td>6.30</td>
</tr>
<tr>
<td>Carboxyglycine</td>
<td>0.0456</td>
</tr>
<tr>
<td>Carboxydiglycine</td>
<td>0.075</td>
</tr>
</tbody>
</table>

$^a$ Recent studies with these derivatives indicate that they may exist in an alternative crystalline modification (lower solubility) in urea solutions. It is believed, however, that the data given here represents the equilibrium with the crystalline form.

Table III
Solubilities of amino acids in aqueous urea solutions

<table>
<thead>
<tr>
<th>Soluble</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g solvent</td>
</tr>
<tr>
<td>Glycine</td>
<td>25.1</td>
</tr>
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<td>Glutamine</td>
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<tr>
<td>Asparagine</td>
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<tr>
<td>Diglycine</td>
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<td>Triglycine</td>
<td>6.30</td>
</tr>
<tr>
<td>Carboxyglycine</td>
<td>0.0456</td>
</tr>
<tr>
<td>Carboxydiglycine</td>
<td>0.075</td>
</tr>
</tbody>
</table>

and to aqueous urea solutions, respectively, and $N_r$ represents the mole fraction at saturation,

$$
\mu_r, u = \mu_r, U + RT \ln \frac{N_r}{N_r} + RT \ln \gamma_r, U
$$

The standard free energy of transfer, $\Delta F$, is defined as $\mu_r, U - \mu_r, W$, and it represents the free energy of transfer from water to the urea solution, at the same mole fraction, at the limit of infinite dilution. By Equation 2,

$$
\Delta F = RT \ln \frac{N_r}{N_r} + RT \ln \gamma_r, U
$$

The first term on the right-hand side of Equation 3 is calculated directly from Table II.

To calculate the ratio $\gamma_r, W/\gamma_r, U$, we have assumed that the interaction between solute molecules in an aqueous urea solution is the same as it would be in pure water, i.e., we have assumed that the values of $\gamma_r, W$ and $\gamma_r, U$ depend only on the solubilities in the corresponding solutions. Since the presence of urea affects the dielectric constant of the solution, this is clearly an oversimplification, but the ratio $RT \ln (\gamma_r, W/\gamma_r, U)$ makes only a small contribution to $\Delta F$, so that the effect of the simplification is negligible.

The activity coefficients have been estimated from the data of Smith and Smith (19). Since their values are on the molality scale, they have been corrected by the relation

$$
\gamma = \gamma (1 + 0.018 m)
$$

where $\gamma$ is the activity coefficient on the molality scale, and $m$ is the molality of the saturated solution, which is readily obtained from the data of Table II. For glycine, alanine, threonine, diglycine, and triglycine, the data of Smith and Smith were used directly. The data for leucine were obtained from Smith and Smith's data for valine by assuming that the difference between log $\gamma$ for leucine and valine is the same as the difference between log $\gamma$ for valine and $\alpha$-amino-isobutyric acid. The values of log $\gamma$ for phenylalanine, tryptophan, and tyrosine were arbitrarily taken as twice the values for leucine, and log $\gamma$ for histidine was taken as equal to log $\gamma$ for leucine. The value of log $\gamma$ for methionine was assumed equal to that of $\alpha$-amino-n-valeric acid, with the observation that $\alpha$-amino-n-butryic acid would have given essentially the same result. The value for asparagine was taken as equal to that for glycine because the parameters which describe the interaction of asparagine with glycine are essentially the same as the parameters which describe the interaction of glycine with itself (20). By analogy, log $\gamma$ for glutamine was assumed equal to log $\gamma$ for alanine. In all cases it was assumed that the racemic mixtures studied by Smith and Smith would not have appreciably different activity coefficients from the $L$ isomers used in the present study. The results of all such estimates are shown in Table III, and the free energies of transfer calculated from the solubilities by Equation 3 are shown in Table IV. From the reproducibility of the solubility measurements, we estimate that $\Delta F$ has a precision of the order of 10 cal per mole for most of the amino acids, so that the activity coefficient corrections made by means of Table III are seen to be not much greater than the uncertainties in the experimental solubilities. For this reason, the assumptions made in arriving at the data of Table III are not critical.

Extensive studies of the solubilities of amino acids and related
For saturated solutions at 25°C:

\[
\Delta F = R T \ln \left( \frac{\gamma_1}{\gamma_2} \right)
\]

For carbobenzoxyglycine and carbobenzoxydiglycine, both of which are uncharged and also have low solubility, the activity coefficients should be close to unity at all urea concentrations.

### Table III
**Values of RT \ln \left( \frac{\gamma_i}{\gamma_2} \right) for saturated solutions at 25°C**

Table presenting values of \( \Delta F \) for different solutes at various urea concentrations.

### Table IV
**Free energies of transfer (\( \Delta F \)) from water to aqueous urea solutions at 25°C**

Table presenting values of \( \Delta F \) for different solutes at various urea concentrations.

Although the interaction of the parent molecules with the solvent must be very different in these three compounds, the effect of adding a side chain is seen to be essentially the same.

The implication of the results given in Table V is that the interaction of the side chain of a branched organic compound with the solvent is approximately independent of the interaction of the backbone to which the side chain is attached. There is no reason to think that this conclusion is unique for the solvents ethanol and water. Careful solubility measurements of hydrocarbon gases in urea solutions made recently by Wetlaufer et al. (22) suggest in fact that the conclusion applies equally well to aqueous urea solutions. In Table VI, for example, we have compared the contribution of a benzene ring and of an isopropyl group to \( \Delta F \) for transfer from water to 8 M urea, first from Wetlaufer's data, in which the group replaces a hydrogen atom on methane, and then from the data of Table IV of this paper, in which the same group replaces a hydrogen atom on alamine. Essentially identical figures are obtained.

Assuming that this same principle applies to all our data, we have calculated the contributions of the amino acid side chains to \( \Delta F \) given in Table VII. These contributions, designated \( \Delta A \), are obtained simply by subtracting from \( \Delta F \) for an amino acid the value of \( \Delta F \) for glycine.

For application of these data to the problem of protein denaturation, it is also of interest to know the contribution of a side chain for a hydrogen atom, which is nearly independent of the kind of molecule to which the side chain is attached. This is illustrated by Table V, which shows the contribution of a non-polar and a polar side chain calculated for the replacement of hydrogen atoms on 3 different parent molecules:

- **Glycine:** \( \cdot \text{H}_2\text{N}-\text{CH}_2-\text{COOH} \)
- **Formylglycine:** \( \cdot \text{H}-\text{CO}-\text{CH}_2-\text{COOH} \)
- **Hydantoin:** \( \cdot \text{HN}-\text{CO}-\text{NH}-\text{CH}_2-\text{CO} \)

### Table V
**Contribution of amino acid side chains (\( \Delta A \)) to \( \Delta F \), for transfer from water to ethanol at 25°C**

Table presenting the contributions of different side chains to the free energy of transfer for glycine, leucine, and aspartic acid.

### Table VII
**Side chain contribution, \( \Delta A \)**

Table listing the contributions of different side chains to the free energy of transfer for glycine, leucine, and aspartic acid.
TABLE VII

Side chain contributions (Δf) to free energy of transfer from water to aqueous urea solutions at 85°

<table>
<thead>
<tr>
<th>Solutes</th>
<th>Δf at urea concentration of:</th>
<th>cal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 M</td>
<td>4 M</td>
</tr>
<tr>
<td>Alanine</td>
<td>0</td>
<td>+15</td>
</tr>
<tr>
<td>Leucine</td>
<td>-110</td>
<td>-155</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>-180</td>
<td>-530</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>-270</td>
<td>-505</td>
</tr>
<tr>
<td>Methionine</td>
<td>-115</td>
<td>-225</td>
</tr>
<tr>
<td>Threonine</td>
<td>-40</td>
<td>-60</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>-225</td>
<td>-395</td>
</tr>
<tr>
<td>Histidine</td>
<td>-100</td>
<td>-160</td>
</tr>
<tr>
<td>Asparagine</td>
<td>-135</td>
<td>-225</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-80</td>
<td>-130</td>
</tr>
</tbody>
</table>

Δf represents the free energy per mole (at infinite dilution) for substituting the amino acid side chain for a hydrogen atom in the urea solution, the amino acid side chain being replaced by a hydrogen in water. The results are independent of the concentrations used for the data of Table IV.

TABLE VIII

Contribution of a glycyl residue (Δf) to free energy of transfer from water to aqueous urea solutions at 85°

<table>
<thead>
<tr>
<th>Solutes used to obtain appropriate Δf values for comparison</th>
<th>Δf at urea concentration of:</th>
<th>cal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 M</td>
<td>4 M</td>
</tr>
<tr>
<td>Diglycine, glycine</td>
<td>-10</td>
<td>-20</td>
</tr>
<tr>
<td>Triglycine, diglycine</td>
<td>-145</td>
<td>-205</td>
</tr>
<tr>
<td>Carboxenzydoglycine, carboxenzydoglycine</td>
<td>-20</td>
<td>-45</td>
</tr>
<tr>
<td>Acetylglcyglycine ethyl ester, butane</td>
<td>-36</td>
<td>-72</td>
</tr>
</tbody>
</table>

Δf represents the free energy per mole (at infinite dilution) for substituting the amino acid side chain for a hydrogen atom in the urea solution. The results are shown in Table VIII, and it is seen that a different value is obtained for the glycyl residue contribution, depending on the molecule into which the glycyl group is inserted. From the two entries of the table in which the glycyl residue represents the first peptide group introduced into the molecule, we get very small values for Δf (less than the difference between Δf for the free glycyl and diglycine we get much larger values (Δf = -310 cal per mole for transfer to 8 M urea)). If no other data were available, and a choice had to be made on the basis of the data of Table IV alone, one would have to choose the higher figure as more representative of the effect of inserting a glycyl residue into a protein polypeptide chain, since it represents insertion of a glycyl residue into a molecule which already has a peptide bond.

Fortunately other data are available from the recent paper by Robinson and Jencks (14), to which reference was made in the introduction. Robinson and Jencks measured solubilities of acetyltetraglycine ethyl ester in water and in aqueous urea solutions, and from their data we have calculated Δf values (in mole fraction units) corresponding to those of Table IV. Because the solute is uncharged, and its solubility is relatively low, the activity coefficient term of Equation 3 can be neglected, giving Δf = -870 cal per mole for transfer to 8 M urea. This value is clearly incompatible with a contribution of the order of -300 cal per mole for each of the glycyl residues. To obtain an actual estimate for Δf, we would need to determine the difference between Δf for acetyltetraglycine ethyl ester and Δf for ethyl acetate. The latter has not been determined, but it is evident from all our data, as well as from Wetlaufer's data on the transfer of hydrocarbons from water to urea solutions (22), that Δf for ethyl acetate will not be a positive number. As an approximation, we may assume that Δf for butane (which would occupy about the same molecular volume as ethyl acetate) can be used. Wetlaufer's data (22) give Δf = -290 cal per mole for transfer of butane to 8 M urea, so that the value of Δf per glycyl residue becomes (for 8 M urea) -145 cal per mole. This value and corresponding values at the other urea concentrations are listed on the last line of Table VIII. It is probable that these figures are more nearly representative of the behavior of glycyl residues in proteins than the figures obtained from our own data. It is evident however that the Δf value for a peptide group is much more sensitive to the nature of neighboring groups than the Δf value for an amino acid side chain, and the use of the figures of Table VIII for the Δf value of a glycyl residue in proteins can represent only an order of magnitude estimate.
It should be noted in this connection that a glycyl residue contains one more hydrogen atom than the repeating backbone unit, —CH—CO—NH—, of a polypeptide chain. Since the side chain contribution to ΔF₁ given in Table VII actually measures the effect of substituting the side chain for a hydrogen atom, the data of Table VII, combined with the side chain contributions of Table VII, will correctly give the contribution of individual amino acid residues to ΔF₁, i.e., in the extent that the ΔF₁ values which we have measured on model compounds truly reflect ΔF₁ values for similar substitutions on protein molecules.

**Choice of Concentration Scale**—Our thermodynamic treatment has been based on the mole fraction scale, so that the ΔF₁ values of Table IV represent free energies of transfer from water to a urea solution, such that the mole fraction of solute remains the same. By virtue of the activity coefficient correction, the values represent extrapolations to infinite dilution of the solute. Our results could equally well have been based on the molality or the molarity scale, and the ΔF₁ values would then have represented free energies of transfer at constant molality or molarity, respectively. Different values of ΔF₁ would have been obtained in this way. Where Xᵥ is the mole fraction of urea in a urea solution (0.185 in 8 M urea), and ρ the density of the urea solution (1.117 in 8 M urea) (23),

\[
ΔF₁ (\text{molarity scale}) = ΔF₁ (\text{mole fraction scale}) + RT \ln (1 + 0.233 Xᵥ)
\]

\[
ΔF₁ (\text{molarity scale}) = ΔF₁ (\text{mole fraction scale}) + RT \ln [(1 + 0.233 Xᵥ)/ρ]
\]

In 8 M urea the differences would amount to +212 and +146 cal per mole, respectively.

The data of principal interest in this paper, however, are the side chain and peptide group contributions to ΔF₁ given in Tables VII and VIII. These are obtained as differences between two ΔF₁ values from Table IV, and are thus clearly independent of the concentration units employed. What has been calculated is the effect of removing a side chain or peptide group from a solute molecule in pure water and replacing it on a solute molecule in a urea solution. This effect (when intersolute interactions have been corrected for) must be independent of concentration, whereas the free energy of transfer of a whole solute molecule from one solvent to another depends, of course, on the concentrations in the two solvents.

**DISCUSSION**

The data of this paper show that the free energy of interaction between predominantly nonpolar side chains of amino acids and water, the so-called "hydrophobic" interaction, is diminished by the addition of urea to the aqueous solution. They also show, however, that the free energy of interaction between the peptide group and the solvent, and between at least some of the polar amino acid side chains and the solvent, also becomes more negative as urea is added. These conclusions agree with those reported by us in a preliminary communication about a year ago (12), and with conclusions reached by solubility studies of other laboratories, e.g., those of Robinson and Jencks (14) and of Wetlaufer et al. (13, 22).

The foregoing conclusions account qualitatively for the well known ability of urea to "denature" or unfold globular proteins. In the typical native globular protein, a relatively small portion of the polypeptide backbone, and relatively few of the nonpolar side chains, are in contact with the solvent. In the unfolded molecule, on the other hand, most of the molecule is in contact with, and therefore interacts with, the solvent. Any change in solvent medium which decreases the free energy of such solvent interactions therefore stabilizes the unfolded conformation relative to the native structure. The principal reason for carrying out the measurements reported in this paper was in fact to provide an explanation for the denaturing action of urea, and a quantitative discussion of that topic, including consideration of all the factors which might be involved in the unfolding of protein molecules, will be given in a separate paper.

Apart from their connection with the problem of protein denaturation, the results of this paper are of direct interest because they are related to the general question of interactions involving water, urea, and the component parts of amino acid or peptide molecules. We consider first the interaction of hydrocarbon side chains with the solvent. The nature of this interaction is well understood as resulting from the inability of a hydrogen-bonded, organized liquid structure to accommodate an inert hydrocarbon moiety without extensive rearrangement of the solvent structure. The rearranged structure (in water solution) may resemble a cage around the hydrocarbon moiety, such as is seen in the crystalline clathrates, e.g., CH₃H₂O, Cl₂H₂O, etc., which have been examined by x-ray diffraction by von Stackelberg et al. (24-26) and by Pauling (27). It should be emphasized, however, that the structures seen in such crystals and the structures which exist when hydrocarbon side chains are in contact with water are not likely to be exactly similar, because the hydrocarbon molecules in clathrate crystals are completely surrounded by a solvent cage, whereas amino acid or peptide side chains are only incompletely surrounded by solvent since there is one direction in which the vicinal space is filled by the —NH—CH—CO— group to which the side chain is attached. Thus structures which are not permitted in the clathrate (because they leave a "hole" on one side of the hydrocarbon molecule) may well be present around hydrocarbon side chains.

Crystalline clathrates between hydrocarbons and urea are as well known as those between hydrocarbons and water (28, 29). Again, either these structures, or modifications thereof, could occur by organization of urea molecules about a hydrocarbon molecule or moiety in an aqueous urea solution. One interpretation of the negative ΔF₁ values for transfer of hydrocarbon molecules or side chains, from water to an aqueous urea solution, could be that urea "cages" indeed replace water "cages" and that they have a lower free energy than water "cages" have. We consider such an interpretation unlikely. The replacement of a cage structure composed exclusively of water molecules by another structure composed exclusively of urea molecules would be a cooperative phenomenon and would result in a nonlinear relation between the side chain contribution to ΔF₁ (Table VII) and the urea concentration. In fact, an almost linear relation exists, suggesting that a variety of mixed structures containing both water and urea molecules may be formed about the hydrocarbon side chains. It is not improbable that the negative free energy of transfer is associated entirely with the ability of the solvent, in aqueous urea solutions, to draw both on urea and on water molecules in accommodating itself to the presence of hydrocarbon moieties, so that a greater adaptability exists than would obtain when only a single kind of solvent molecule were available.
A similar question of interpretation bears on the data of Table VIII. Does the stabilization of the peptide group by urea reflect the creation of association products between urea and the peptide group in place of the peptide hydrates which presumably exist in water? Or is the stabilization simply the result of another kind of hydrophobic effect? It is possible, for instance, that the peptide group is easily soluble in water because of an energetically favorable association with water, but that the water in the association product is so oriented that it cannot readily fit in with the normal structure of liquid water. The availability of urea molecules may help to create a superior adaptability of the solvent structure near the hydrated peptide group without affecting the hydrogen bonding between the peptide group and 1 or more water molecules.

Thermodynamic data alone cannot with any certainty choose between these possibilities. It is possible to show, however, that the stabilization of the glycyl residue by urea is a general phenomenon applicable to terminal amide groups and even to urea itself, as well as to the peptide group. This is shown by the data of Table IX.

Despite the general uniformity of these data, we feel that they are not necessarily in conflict with the conclusion, reached from spectral data by Klotz and Franzen (11), that hydrogen bonds between peptide or amide groups and water are much stronger than interpeptide or interamide hydrogen bonds.

It is evident from the foregoing discussion that these data cannot be used to decide whether the interpeptide and other hydrogen bonds which are known to be present in globular proteins make a positive or negative contribution to the free energy of unfolding of the globular structure; i.e., they cannot be used to obtain a value for \( \Delta F \) for the peptide group in the general equation for the free energy of unfolding published by one of us recently (8).

### SUMMARY

The solubilities of 11 amino acids, two peptides, and two carbobenzoxy derivatives have been determined in water, 2 m urea, 4 m urea, 6 m urea, and 8 m urea, at 25°. The data have been treated so as to yield the effect of urea on the free energy of solvent interaction which accompanies (a) the insertion of a glycyl residue into an existing molecule and (b) the substitution of an amino acid side chain for a hydrogen atom on the glycyl residue. The sum of these free energies should be approximately equal to the free energy which accompanies insertion of an amino acid residue into a protein molecule.

Nonpolar side chains were found to give rise to increased solubility in urea solutions, so that the free energies of solvent interaction attributable to these side chains become more negative as urea is added. The maximal effect was observed for tryptophan, for which, in 8 m urea, \( \Delta F \) = -920 cal per mole.

Polar side chains containing amide groups also give rise to increased solubility in urea solutions, and so does insertion of a glycyl residue. The actual change in free energy of solvent interaction attributable to insertion of a glycyl residue depends on the nature of the molecule into which the residue is inserted, so that a value for insertion into a protein chain could not be determined unequivocally. A value of \( \Delta F = -145 \) cal per mole (in 8 m urea) was concluded to be a reasonable estimate, but it must be considered subject to revision.

The data as a whole account for the denaturating action of urea on globular proteins as being due to stabilization of the unfolded form of a protein molecule, both by diminishing the hydrophobic interaction between nonpolar groups and water and by increasing the affinity of the solvent for amide and peptide groups.

### ACKNOWLEDGMENT

We wish to acknowledge the skillful technical assistance which we have received from Mrs. P. M. Hudson.

### REFERENCES

The Solubility of Amino Acids and Related Compounds in Aqueous Urea Solutions
Yasuhioko Nozaki and Charles Tanford


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