Solvent and Conformational Effects on the Ultraviolet Spectra of Polypeptides and Substituted Amides

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(Received for publication, June 16, 1962)

The study of the ultraviolet spectra of proteins and polypeptides shows changes in the 215- to 245-\(\mu\) region, occurring simultaneously with alterations in conformation, such as denaturation or helix-coil transitions (1, 2). Recently (3-5), the rotary dispersion curves of helical synthetic polypeptides and certain native proteins have been extended into the wave length range mentioned above and shown to exhibit rotary anomaly in the form of a Cotton effect centered at approximately 225 \(\mu\). This anomaly is absent in the randomly coiled or denatured materials. It has been suggested (1, 3-7) that both these phenomena are connected with an \(\pi-\pi^*\) transition in the spectrum of the peptide bond.

The spectra of carboxylic acids and simple amides show a shoulder, which has been assigned to such a transition (8), appearing on the long wave length side of the intense \(\pi-\pi^*\) peak in the far ultraviolet. Some evidence for its existence has been derived also from the polarized electronic spectra of crystalline myristamide (9). In the spectra of small peptides, this transition does not appear (10). The dichroism in the 220-\(\mu\) region in the polarized spectra of oriented films of helical polypeptides is, however, suggestive of the presence of an \(\pi-\pi^*\) band (7).

The position of \(\pi-\pi^*\) bands has generally been found to vary with the polarity of the solvent used. In particular, these bands undergo blue shifts, i.e. shifts to shorter wave lengths, when a hydrocarbon solvent is replaced by a hydroxyllic one (11). This property might provide a test for the presence of such a band in the spectrum of the peptide group. The same effect could also conceivably lead to the appearance of the difference spectrum of native versus denatured proteins in the region considered (1), since, as has been conjectured (12, 13), the effective medium might be very different with respect to polar character in the two cases.

It was felt, therefore, that the study of the influence of solvents of varying polarities on the spectra of polypeptides and simple, substituted amides could yield some further information on the involvement of an \(\pi-\pi^*\) transition in the phenomena described here.

EXPERIMENTAL PROCEDURE

The polypeptides were prepared by polymerization of the corresponding \(N\)-carboxy amino acid anhydrides by the usual procedures (14). Poly-L-lysine-HCl and poly-L-glutamic acid were high molecular weight preparations with an average degree of polymerization of several hundred. Polysarcosine had an average degree of polymerization of 30. \(N\)-Methylacetamide and \(N,N\)-dimethylacetamide were Eastman Kodak white label products. Methanol was spectro quality from Eastman Kodak. p-Dioxane and acetonitrile were analytical grade (purissima) solvents manufactured by Fluka A-G, Switzerland. Cyclohexane was the spectro quality British Drug Houses reagent. Perchloric acid, 70\% in water, was the Analar product from British Drug Houses. The concentrations of the solutions of amides and polypeptides were determined by micro-Kjeldahl procedure.

Spectra were measured on a Beckman model DK-1 recording spectrophotometer with calibrated 1-, 2-, and 10-mm Supersil cells with stoppers. Stray light was kept at a minimum by working at narrow slits and at low concentrations, and by stopping runs well in advance of the solvent cut-off, as judged by the slit width variation. The error, due to stray light, at the low wave length limit, was about 2\% of the measured absorbancy values.

In a number of preliminary experiments, difference spectra were obtained directly by the tandem cell technique. However, this was unsatisfactory because, owing to the absorbancy of the reference cell tandem, the cut-off point was moved up to longer wave lengths. In consequence, difference spectra were computed from the spectra of the solutes run against the appropriate solvent blanks.

RESULTS

Aqueous Solutions—\(N\)-Methylacetamide and \(N,N\) dimethyl acetamide were chosen as simple models since they contain the amide bond as the sole chromophore in the region of interest. Fig. 1a shows a comparison of the spectra of NMA\(^1\) and of poly-L-lysine in aqueous solution at pH 5, when the polymeric base is fully ionized and in the randomly coiled form. The spectrum of ionized poly-L-glutamic acid can be essentially superimposed on that of ionized poly-L-lysine. Fig. 1b gives the corresponding spectra for DMA and polysarcosine. From the above comparison, it is evident that the polypeptide spectra, and especially that of poly-L-lysine, are substantially shifted to the red with respect to those of the corresponding amides. This is, however, not a consequence of the polymeric nature of these materials, but is due rather to the presence of the relatively large side chains, as is indicated in observations on the spectra of dipep-
tides of various amino acids (10), and also from the hyperchromicity at 205 m\(\mu\) found in \(N\)-substituted acetamides, when the size of the substituent group is gradually increased (15). As an example, the spectrum of \(N\)-leucylglycylglycine from Saidel's data (10) is reproduced on Fig. 1a and found to be similar to that of the polymer. In agreement with this view, the smaller displacement of the polysarcosine spectrum relative to that of DMA reflects the greater similarity in substitution of these materials.

Organic Solvents—Figs. 2a and 2b show the spectra of NMA and DMA in water, methanol, acetonitrile, dioxane, and also 98% cyclohexane in the case of NMA. The direction of the shift is that expected for an \(n\rightarrow\pi^*\) transition. The spectrum of NMA in cyclohexane is shifted to the blue relative to those in acetonitrile and dioxane, rather than exhibiting the red shift expected on the basis of refractive index and hydrocarbon character. A clue to this behavior may be found in the marked decrease in absorbancy below 215 m\(\mu\) occurring in this spectrum, which suggests that this solvent strongly affects the position and perhaps, also, the intensity of the \(\pi\rightarrow\pi^*\) maximum. Some of the features of the difference spectra in the various solvents, relative to that in water, are set out in Table I.

Perchloric Acid—Protonation of the absorbing species has been shown to lead to the disappearance of \(n\rightarrow\pi^*\) bands (11). Spectra of the amides, run in 2 M perchloric acid, resulted, however, in changes which seemed too large to be accounted for solely in terms of the disappearance of an \(n\rightarrow\pi^*\) band. With both NMA and DMA, the intensity decreased drastically over the entire spectrum, down to 190 m\(\mu\) (this wave length could be reached in this case, owing to the good transmittance of the solvent). For DMA, the maximal difference in intensity between the aqueous and the perchloric acid solutions occurred at about 205 m\(\mu\) and was of the order of \(\Delta\varepsilon \sim 2000\). In contrast

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**TABLE I**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>(\Delta\varepsilon_{\text{max}})</th>
<th>(\lambda_{\text{max,\text{HCl}}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMA</td>
<td>Methanol</td>
<td>222</td>
<td>30</td>
</tr>
<tr>
<td>NMA</td>
<td>Acetonitrile</td>
<td>224</td>
<td>69</td>
</tr>
<tr>
<td>NMA</td>
<td>Dioxane</td>
<td>224</td>
<td>73</td>
</tr>
<tr>
<td>NMA</td>
<td>Cyclohexane*</td>
<td>227</td>
<td>44</td>
</tr>
<tr>
<td>DMA</td>
<td>Methanol</td>
<td>227</td>
<td>93</td>
</tr>
<tr>
<td>DMA</td>
<td>Acetonitrile</td>
<td>232</td>
<td>118</td>
</tr>
<tr>
<td>DMA</td>
<td>Dioxane</td>
<td>232</td>
<td>129</td>
</tr>
</tbody>
</table>

* Cyclohexane-dioxane, 98%:2% (volume for volume).
† Crossover point outside measured range.
solvent-versus-water difference spectra of the polymers in the random-coil form are thus significantly different from that of NMA in the same solvent versus water (Curve 4, Fig. 3). In the case of the latter, a red shift is observed, giving a positive difference spectrum. At pH values of approximately 4, at which poly-L-glutamic acid in water is essentially in the fully helical form (17, 18), the 50% dioxane-versus-water difference spectrum (Curve 3, Fig. 3) shows a red shift of roughly the same magnitude as that of NMA, as can be seen from the similar heights of the respective peaks. The displacement between the two difference spectra, with respect to wave length, seems to mirror the displacement in the spectra of amide and polymer, mentioned previously (see Fig. 16).

Fig. 3 gives also the difference spectrum obtained by comparing poly-L-glutamic acid in the helical form (pH 4) with the same in the randomly coiled form (pH 9), both in aqueous solution (Curve 1). The A peak per residue obtained is approximately 110 and Amax = 225 μm. Poly-L-lysine gives a similar result. It is seen that the intensity of the helix-coil difference peak in water is far higher than that of the solvent difference peaks in 50% dioxane-water.

**DISCUSSION**

As mentioned previously, the long wave length shoulder appearing in the 205- to 215-μm region in the spectrum of carboxylic acids and unsubstituted amides is usually not apparent in that of di- and tripeptides. It has been suggested (8) that this is due to the bathochromic effect, occurring on substitution, which is stronger for the intense π-π* than for the weak n-π* band. The n-π* band is thus practically hidden beneath the strong π-π* band. Our results indicate that the n-π* band can still be partially resolved in the spectra of NMA and DMA by using the solvent-induced shifts. If it is assumed that the organic solvents do not induce any drastic change in the intensity of the n-π* transition, then the crossover points between the organic solvent and the aqueous spectra provide an estimate of its wave length of maximal absorption in the aqueous solution spectrum. These points will lie at longer wave lengths than A in water, regardless of whether the position of the neighboring π-π* band remains unchanged or is shifted to the blue in the organic solvent (11), since, in the latter case, the spectra would cross even further to the red. The maximum of the n-π* band in aqueous solutions of NMA thus lies at approximately 210 μm, indicating little change from its position in unsubstituted acetamide. It would seem surprising that monosubstitution on the nitrogen should not have affected the position of this band more appreciably, especially since, from the similarity of the solvent effects in NMA and DMA, one must draw the conclusion that the n-π* transition does undergo a red shift, comparable in magnitude to that of the π-π* band, on disubstitution. The cause for this apparent inconsistency could lie in a difference in the strength with which these molecules interact with the solvent. A comparison of the unperturbed positions of the band could possibly be obtained from vapor spectra of NMA and DMA, but this is of no immediate relevance to the purpose of the present study.

To discuss now the striking difference noted before, with respect to the solvent effect, between the spectra of the random-coil and the helical forms of the polymers, it should be remembered that all the polymer spectra are substantially shifted to
the red with respect to that of NMA (Fig. 1a), an effect which has been attributed mainly to the substitution on the $\alpha$-carbon. The maximum of the $\pi-\pi^*$ band of the randomly coiled polymer in water lies at 192.5 $\text{nm}$ (2), i.e. at least 7.5 $\text{nm}$ farther to the red than that of NMA. One possible explanation for the absence, in this case, of a red shift, such as that found for NMA in 50% dioxane-water, could be an invariance of the position of the $n-\pi^*$ band in the aqueous spectrum with respect to this kind of substitution. This probably would bring the two bands into complete superposition, so that a blue shift of the $\pi-\pi^*$ band, as is apparent in the random-coil polypeptide spectra in Fig. 3, would tend to compensate the red shift of the $n-\pi^*$ band over its entire width. Whatever the true cause of this observation, the reappearance of a red shift in the helical polypeptide may be taken as an indication that, owing to the conformational transition, the $n-\pi^*$ band has been sufficiently altered in position or intensity to overcome the masking effect of its intense neighbor. This alteration appears also to be the origin of the helix-coil difference peak in aqueous solution, since it is precisely this spectral region that is solvent-sensitive. The $\Delta \epsilon_{\text{max}}$ and $\lambda_{\text{max}}$ values of the helix-coil difference spectrum would indicate that a net red shift of the $n-\pi^*$ band of approximately 10 $\text{nm}$ is involved in the conformational transition, but changes in the intensity of the band might appreciably affect this estimate. The intensity increase is probably at least partly due to an enhanced intensity of the $n-\pi^*$ band, in view of the fact that the large decrease in oscillator strength of the $\pi-\pi^*$ band, occurring on helix formation (2, 19, 20), would otherwise reduce the absorption intensity also in the region concerned.

It may be mentioned again that the spectra of poly-L-lysine and poly-L-glutamic acid are practically identical for the random form as well as the helical form respectively and, therefore, the spectral change appears to be independent of the nature of the ionizing group present and the pH of the solution. It may also be worthwhile to comment on the relatively small displacement, involving a red shift of approximately 2.5 $\text{nm}$, between the spectra of DNA and poly-L-sarcosine. Owing to its inability to form intramolecular hydrogen bonds, poly-L-sarcosine is almost certainly in a random conformation and thus the peptide groups are free to take up all possible orientations. The structural similarity between DNA and the repeating unit of the polymer affords a direct comparison in this case, without complications due to difference in substitution, of the respective spectra. Hence, the observed shift can be interpreted as an expression of the interaction between neighboring peptide groups, and its smallness as an indication of the smallness of this interaction. A similar conclusion has been reached from the study of oligopeptides of glycine (8). However, this seems no longer to be true when the peptide groups are arranged in a definite steric relationship to one another, such as that provided by the $\alpha$-helical conformation.

When considering the optical changes in the 225-$\text{nm}$ region as a whole, one cannot fail to observe certain similarities to recent findings on the optical properties of a series of unsaturated ketones (21-23), which all show intensified, red-shifted carbonyl $\pi-\pi^*$ bands in the 300-$\text{nm}$ region, associated with large Cotton effects. Theoretical work relates both the enhanced intensity (25) and optical activity (26) to mixing of double bond $\pi$ and carbonyl $\pi^*$ and $n$ orbitals, in cases of favorable steric configuration, leading to transfer of intensity from the strong to the weak band. If the same process can take place between the respective transitions of peptide groups, the helical conformation might be looked at as serving to fix a suitable geometry whereby the $n-\pi^*$ band of a given group can pick up intensity from the $\pi-\pi^*$ band of its neighbors. (This cannot occur within the same peptide group because of the orthogonality of the respective orbitals.) The near neighbors might be expected to have different contributions to the sign of rotation and rotational strength. However, the overall effect cannot simply be assessed as a sum of pairwise interactions. The possibility that such intensity transfer occurs in polypeptides is purely speculative at present, but an evaluation of its possible contribution to the optical changes observed might perhaps be warranted at some stage in future theoretical treatments.

Finally, the data presented in the foregoing suggest that the difference spectra, obtained on comparing proteins in the native and denatured state (Table II) may be largely attributed to differences in helical content between the two forms, rather than to environmental changes. The $\Delta \epsilon$ per residue values listed for the difference spectra of the native versus the denatured proteins are greater than can be expected on the basis of the solvent effects found alone, if poly-L-glutamic acid is taken as a reference. Thus, for instance, for the albumins, having a helical content of roughly 50% (27, 28), the evaluation of the solvent contribution to the optical changes observed might perhaps be warranted at some stage in future theoretical treatments.

\begin{table}
\centering
\caption{Values of $\Delta \epsilon$ per residue obtained from difference spectra of protein in neutral solution (sample cell) and in acid (reference cell). Data taken from Reference 1.}
\begin{tabular}{lllll}
\hline
Protein & Residues & $\Delta \epsilon_{\text{max}}$ & $\Delta \epsilon$ & $\Delta \epsilon$/residue \\
\hline
Bovine serum albumin & 588 & 234 & 31,090 & 52 \\
Human serum albumin & 526 & 233 & 29,250 & 55 \\
Papain* & 185 & 235 & 11,450 & 62 \\
Rhinomelanea & 154 & 234 & 3,040 & 24 \\
Insulin & 51 & 235 & 2,400 & 48 \\
\hline
\end{tabular}
\begin{tablenotes}
* An error was made in the value computed (1) for $\Delta \epsilon$/residue (120) for papain.
\end{tablenotes}
\end{table}

The magnitude of the Cotton effect, as observed by Simmons et al. (3, 5), Blout (4), and lately also by Jirgensons (24), falls within the range of amplitudes of the Cotton effects found in the ketones concerned, i.e. $10,000$ to $40,000$, peak to trough (22).
protein difference spectra. The displacement of the protein difference peak to somewhat longer wave lengths could, possibly, be a consequence of the environmental effect. It has, however, not yet been possible to evaluate in detail the contribution of changes in the absorption spectra of the various amino acid side chains present.

**SUMMARY**

The effect of solvents of varying polarities on the ultraviolet spectra of N-methylacetamide and N,N-dimethylacetamide is consistent with the presence of an $n-\pi^*$ transition at the long wave length edge of the main absorption peak.

In the spectra of poly-L-glutamic acid and poly-n-lysine in the random-coil form this transition cannot be resolved in the same way, owing presumably to a superposition of substitution and solvent effects. The spectra of poly-L-glutamic acid in the helical form and polysarcosine do show solvent-induced shifts resembling those observed with N-methylacetamide and N,N-dimethylacetamide, respectively.

The spectral change caused by the helix-coil transition in aqueous solutions of poly-L-glutamic acid and poly-L-lysine cannot be accounted for exclusively in terms of the medium effects observed, because of its greater magnitude. From the solvent effect on the spectrum of the helical polypeptide, it is concluded that the $n-\pi^*$ transition is directly involved in the generation of the helix-coil spectral change. The suggestion is made that the latter may result from a red shift and intensification of this transition, due to favorable steric overlap of the electronic orbitals involved, when the polymer is in the helical conformation.

**Acknowledgment**—We wish to thank Dr. A. Berger for helpful discussions and Dr. L. E. Orgel for valuable criticism of the manuscript.

**REFERENCES**

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