the substitution of D for H in the enzyme alters the secondary and tertiary structure of the enzyme is not clear. We are at present investigating this question.

This and other deuteriated enzymes may serve as useful tools for study of the mechanism of enzyme action.

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† Medical Student Investigator, supported by the Health Research Council of the City of New York.
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THE FAR ULTRAVIOLET ABSORPTION SPECTRA OF POLYPEPTIDE AND PROTEIN SOLUTIONS AND THEIR DEPENDENCE ON CONFORMATION

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It has been predicted1, 2 on the basis of exciton theory that the parallel alignment of peptide chromophores prevailing in the helical conformation of a polypeptide chain would give rise to a hypochromic effect in the absorption band associated with these chromophores, as well as a splitting of this band into two components separated by some 2,800 cm\(^{-1}\) and polarized at right angles to one another. This hypochromicity was first observed by Imahori and Tanaka3 in poly-L-glutamic acid when the polymer was in its \(\alpha\)-helical form at a pH at which the carboxyl groups are protonated. When the pH is raised, these groups ionize and a helix-random coil transition occurs, in the course of which the hypochromicity vanishes, with a consequent increase of some 65 per cent in the absorbance at 190 m\(\mu\).

As has previously been briefly reported,4 we have observed a shoulder at about 205 m\(\mu\) in the spectra of both poly-L-glutamic acid and poly-L-lysine in the helical form, which may be presumed to arise from the predicted exciton splitting. This has also been noted by Tinoco et al.5 in poly-L-glutamic acid spectra obtained with a vacuum spectrograph, though not in spectra of poly-L-lysine measured with a photoelectric instrument; neither was it observed by Imahori and Tanaka3 who used a similar instrument for poly-L-glutamic acid. It would appear that the failure of these workers to detect this feature was due to instrumental shortcomings, possibly poor stray light elimination. All the spectra discussed in the present paper were measured with a Beckman DK-2 recording instrument.
It seems clear from the work of Imahori and Tanaka that the absorbance of poly-L-glutamic acid under varying conditions of pH and ionic strength can be used to give a direct estimate of helical content. It is of interest to establish whether this technique is generally applicable to polypeptides and to what extent it may be further applied to proteins, and to elucidate in general terms the factors contributing to the absorption spectra of proteins in this region, which has hitherto been largely unexplored.

**Experimental.**—The necessity of using a solvent of low absorption in the 190 μ region restricted the choice of polypeptides for the study of the helix-coil transition to those which undergo the conformational transition as a result of changes in pH. The influence of changes in temperature at constant pH was also studied.

**Polypeptides:** Poly-L-lysine HBr was dialyzed exhaustively against 0.01 N HCl in order to replace the highly absorbing bromide ion by chloride ion, which has a molar extinction coefficient at 190 μ of no more than about 1,000. The resulting hydrochloride, after further dialysis against distilled water, was recovered by lyophilization. Two preparations were made, having molecular weights of about 30,000 and 300,000 respectively, as estimated from viscosity measurements in 0.2 M sodium chloride. Poly-L-glutamic acid was prepared from poly-γ-benzyl-L-glutamate bought from Pilot Chemicals Inc. and had a molecular weight of about 80,000.

Poly-D,L-glutamic acid was prepared by polymerization of γ-benzyl-D,L-glutamate N-carboxy anhydride in benzene using n-hexylamine as initiator and subsequent debenzylation, followed by dialysis and lyophilization. The molecular weight of the benzyl ester of the polymer was about 30,000 as estimated from viscosity measurements in dichloroacetic acid. Examination of the absorption spectra of all polypeptides in the 260 μ region showed them to be free of protecting groups. Amino acids and dipeptides were obtained from either National Biochemical Corporation or Mann Research Laboratories and dried to constant weight in vacuo.

**Proteins:** Paramyosin was prepared by B. S. Harrap from clam adductor muscle. Ribonuclease was purchased from Worthington Biochemical Corporation and oxidized according to the procedure of Harrington and Schellman. β-Lactoglobulin was a product of Pentex, Inc., used without further purification. Beef insulin was the five times recrystallized product of Lilly Research Laboratories, Lot T-2 42. Crystalline sperm whale myoglobin was a gift of J. C. Kendrew.

**Preparation of solutions:** Stock solutions, the concentrations of which had been determined by Kjeldahl nitrogen analysis, were diluted to give absorbances of about 0.5 in 2 mm cells. The pH of the polypeptide solutions was adjusted with HCl or NaOH. In the low pH range, the reference solutions were made up with equal concentrations of chloride ion in the form of HCl or NaCl without adjustment of pH. At high pH, however, the pH in both cells was adjusted to the same value, because in this range the absorption of the hydroxyl ion becomes significant. The molar extinction coefficient of hydroxyl ion at 190 μ being about 4,500, a solution of pH 11 in a 2 mm cell has an absorbance of about 1.0 from the hydroxyl ions alone. A discrepancy of 0.03 pH units in this range therefore causes a change of about 0.04 units in absorbance: this results in an error of about 10% in the calculated molar extinction coefficient of the polypeptides. It was therefore necessary to exercise the greatest care in controlling the pH in both cells and protecting the solutions from the atmosphere, by working in stoppered cells.

Experiments with some simple amines showed that the absorption of both the ionized and the un-ionized amino group is negligible. The same is true for the un-ionized carboxyl group. The molar extinction coefficient of the carboxylate ion at 190 μ is about 1,000, and suitable corrections were applied to the calculated extinction coefficients in the case of the polyglutamic acids. Both the SO₄⁻ and the H₂PO₄⁻ ion have sufficiently low absorption to make their use possible in concentrations up to about 0.01 molar. In the paramyosin and insulin experiments, 0.01 M sulfuric acid was used to adjust the pH; the β-lactoglobulin was dissolved in phosphate buffer.

The contribution of the side chains to the absorption of the proteins was calculated making use of published analytical data. For paramyosin, the average values of several analyses of Venus mercenaria tropomyosin were taken from data of Komintz et al. For insulin and ribonuclease, the compositions taken from the published amino acid sequences were used. For β-lactoglobulin, the analyses of Stein and Moore were used, and for myoglobin those of Edmundson.
It was assumed in the first instance that the extinction coefficients and absorption maxima of the various side chain chromophores were all unchanged when incorporated in the protein.

Spectra: The spectra were recorded on the Beckman DK-2 spectrophotometer. This instrument, as specially adapted for the far ultraviolet, is equipped with selected synthetic quartz optics to provide high transmittance in the short-wavelength ultraviolet region. The hydrogen arc and the RCA 7200 photomultiplier have fused quartz envelopes, and oxygen is eliminated as far as possible from the light path by flushing nitrogen through the instrument. Before starting the measurements, nitrogen (water-pump grade) was passed through a silica gel drying tube and into the instrument at a rate of 30-40 cu. ft./min until the slit width at any point in the region of oxygen absorption (below about 200 m\(\mu\)) had decreased to a constant value, indicating that the oxygen concentration in the light path had reached a stationary minimum. The same rate of flow was then maintained throughout the measurements.

Cells: All cells were of "Supersil" grade quartz and 2 mm path length, supplied by either Pyrocell Manufacturing Co., or Quaracell Inc. When properly cleaned by detergent or boiling nitric acid, these cells had excellent transmittance down to the limit of the instrument at about 180 m\(\mu\). Ground glass- or silicone rubber-stoppered cells were used to protect the aluminized mirrors of the optical system from contamination by solvent vapors. In the heating experiments, the use of cells with well-fitting ground glass stoppers is imperative. Loss of solvent was in this case found to be no more than a few per cent at most. The 2 mm cells were calibrated for path length by comparison with 1 cm cells, and corrections were applied in the calculation of molar extinction coefficients.

In the heating experiments, a modified Beckman temperature-regulated cell block was used in conjunction with a circulating heating bath.

Solvent water was redistilled in a Pyrex still. Degassing by boiling was found to have very little effect on the transmittance and was considered unnecessary. In the 2 mm cells, meaningful measurements could be made down to 183 m\(\mu\), the slit width remaining below 0.5 mm under proper operating conditions. Owing to broadening of the water absorption band, the cutoff moves to longer wavelengths as temperature is increased, and at 90° it lies at about 187 m\(\mu\).

The use of D\(\text{2}O\) as solvent extends the working range by 2-3 m\(\mu\) toward shorter wavelengths.

Stray light: Stray light becomes a factor of the greatest importance in spectrophotometry in the wavelength range here considered. The particular virtues of the Beckman DK-2 instrument in this respect are demonstrated by the data given by Stich et al.\(^{11}\) Stray light tests were carried out at intervals according to the procedure suggested by Beaven and Holiday.\(^{12}\) Either a Corning 7910 filter cutting off at about 220 m\(\mu\) or a 1 cm cell filled with spectroscopically pure isopropanol, which cuts off at somewhat lower wavelength, was placed in the sample beam. Any energy reaching the detector below the cutoff wavelength may then be regarded as stray light. The value so obtained is probably somewhat smaller than the true one, but it is clear from Figure 1 that the instrument will give valid results at 185 m\(\mu\). Additional tests for adherence to Beer's law were performed, and it was concluded that under optimal operating conditions with adequate nitrogen flushing errors due to stray light are not significant up to total absorbance values (solute and solvent) of about 1.0.

Macromolecular scattering: The contribution of scattered light to the absorbance of polypeptides of high molecular weight was roughly estimated from a logarithmic plot of absorbance versus wavelength in the range 350 to 250 m\(\mu\), where there are no absorption bands.\(^{13}\) The linear plots were extrapolated into the region of absorption, and it was clear on this basis that the contribution of macromolecular scattering at these concentrations was negligibly small, and indeed comparable with errors in absorbance readings.

Fig. 1.—Stray light level in the Beckman DK-2 recording spectrophotometer. Transmittance of isopropanol in 1-cm path length at short wavelengths.
Infrared spectra: Infrared spectra in D₂O solution were determined with a Perkin-Elmer 21 Spectrophotometer, using a calcium fluoride cell of 0.025 mm pathlength.

Polypeptides.—Poly-L-lysine: The far ultraviolet spectra of poly-L-lysine in three different conformations is shown in Figure 2. The full line refers to the randomly coiled, or disordered, form: it is single peaked with a molar extinction coefficient of about 7,100 at the maximum, which is located at 192 μm. When the pH is changed from neutrality, where this result was obtained, to 10.8, the polypeptide takes up the α-helical conformation, and this is seen to be accompanied by a substantial change in the spectrum. There is a large hypochromic effect, the maximum extinction coefficient being reduced to 4,400, together with the appearance of a shoulder at 205 μm. At about 215 μm, the curves cross and they meet again at 240 μm, where the absorbance vanishes.

![Graph of ultraviolet absorption spectra](image)

**FIG. 2.**—Ultraviolet absorption spectra of poly-L-lysine hydrochloride in aqueous solution: random coil, pH 6.0, 25°; helix, pH 10.8, 25°; β-form, pH 10.8, 52°.

It has been found that upon heating solutions of poly-L-lysine in the α-helical form there is a complete and irreversible conversion to the β-form. At moderate concentration, a precipitate forms; at the high dilution employed here, only limited molecular association occurs. The transition to the β-form is readily shown by the shift of the infrared amide I band from 1,638 to 1,610 cm⁻¹ in D₂O solution (Fig. 3). The ultraviolet spectrum changes markedly as well, as shown by the other dashed line in Figure 2. The hypochronism is lost, the peak shifts to 194 μm, and the absorbance is seen to be somewhat higher than for the disordered form at all higher wavelengths.

While the spectrum for the β-form is not sufficiently different from the disordered form to be useful diagnostically, the much larger differences between the disordered form and the helical form should prove useful, particularly since infrared spectra cannot readily differentiate these two forms.
Poly-L-glutamic acid: The spectra for the randomly coiled and α-helical forms of this polypeptide are shown in Figure 4. The spectrum for the random form has been corrected for carboxylate absorption. Again, the choice of pH and the consequent state of ionization dictates the conformation. The marked hypochromism of the helical form is evident, as first observed in this material by Imahori and Tanaka. However, in contrast to their earlier observations a splitting is seen here, as in poly-L-lysine, into a maximum located at 190 and a shoulder at 205 m.μ.

It has been shown previously that the heating of poly-L-glutamic acid in the helical form causes some conversion into the randomly coiled form. When the temperature is raised in this case, there is seen a change toward the spectrum of the randomly coiled form as expected (Fig. 4).

By converting the absorbance at the peaks of the spectra to molar extinction coefficients, values of 7,100 and 4,200 are found. These compare closely with those for the corresponding forms of poly-L-lysine and indicate that the hypochromic effect is associated with the chain conformation and is independent of the side chain.

Poly-D,L-glutamic acid: Studies on polypeptides made from racemic mixtures indicate that like residues tend to cluster in the chain giving rise to marked departure from random sequences. If this is so, poly-D,L-glutamic acid may contain some helical regions at acid pH, where it is protonated. Such helical regions would not be detectable from rotatory dispersion measurements, since there would be equal amounts of essentially compensating types, that is, right-handed L-residue helices and left-handed D-residue helices. Consequently, it was of interest to examine the ultraviolet spectra of poly-D,L-glutamic acid to see if in acid solution it was hypochromic with respect to the randomly coiled form that occurs above pH 5.

The results are shown in Figure 5. The upper curve refers to the randomly coiled form, and the dashed curve represents the same curve after correction for carboxylate ion absorption. The spectrum at lower pH, where the helical form is stable in poly-L-glutamic acid, is seen to exhibit only modest hypochromism. Relative to the peak at pH 8.4, the peak at pH 3.0 is diminished by only 10 per cent in comparison to nearly 40 per cent for corresponding conditions in poly-L-glutamic acid. This evidence would therefore indicate that poly-D,L-glutamic acid when not ionized exists in some measure in the form of short helical regions, not detectable by optical rotatory methods.
Characteristic spectral differences near 230 m\(\mu\): The higher absorbance of the helical form from 220 to 240 m\(\mu\) has already been noted. The possible origins of absorption in this region are discussed in the following paper.\(^2\) At this juncture, we merely wish to point out that this relatively small difference, which apparently depends on conformation, can be exploited by the use of difference spectroscopy. By the use of high absorbances at 230 m\(\mu\) and a solution of the randomly coiled polypeptide in the reference cell, it is possible to follow helix-coil transitions by varying the pH in the sample cell. This procedure makes possible the use of many reagents opaque at 190 m\(\mu\) and permits the use of the ultraviolet technique for studying conformation with instruments not fitted with “Supersil” optics. More-
the same in the protein as in the amino acid, even though the environment and interactions are different in the two cases. In the absence of data on model compounds to test the validity of this assumption, it is instructive to compare the results obtained by the present method with quantitative structural information from other sources. On the further assumption that the residues of the protein molecule are distributed between the random and the helical states only, the fraction of residues in helical conformation is given by

$$f = \frac{\sum n_i \epsilon_i + N \epsilon_r - A/c}{N(\epsilon_r - \epsilon_h)},$$

where $\sum n_i \epsilon_i$ is the sum of the side chain extinction coefficients, $\epsilon_r$ and $\epsilon_h$ the residue extinction coefficients of the random and helical form respectively at a given wavelength, $N$ the number of peptide bonds, $c$ the concentration in moles per liter, and $A$ the absorbance measured for a 1 cm path length.

The only quantitative data hitherto available on the absorption spectra of amino acids below about 200 m\(\mu\) are due to Platt and Klevens. Since these authors give only a selection of the natural amino acids, we have determined the spectra for a number of other amino acids in the Beckman DK-2 spectrophotometer down to about 185 m\(\mu\). The molar extinction coefficients at these wavelengths in the region of peptide absorption are set out in Table 1.

The spectra of several proteins are shown in Figures 6 and 7. The maxima are seen to lie at about 190 m\(\mu\) in all cases. Furthermore, conditions which generally lead to more unfolded conformations, such as heat treatment or oxidation of disulfide bridges, are seen to be associated with increased absorbance.
TABLE 1

Molar Extinction Coefficients of Chromophoric Amino Acid Side Chains

<table>
<thead>
<tr>
<th>Side chain</th>
<th>190 mμ</th>
<th>197 mμ</th>
<th>205 mμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylate ion</td>
<td>900</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>Methionine</td>
<td>1,600</td>
<td>1,600</td>
<td>1,600</td>
</tr>
<tr>
<td>Cystine(^)</td>
<td>2,500</td>
<td>1,500</td>
<td>1,000</td>
</tr>
<tr>
<td>Histidine</td>
<td>5,300</td>
<td>3,600</td>
<td>4,200</td>
</tr>
<tr>
<td>Amide</td>
<td>6,200</td>
<td>1,400</td>
<td>450</td>
</tr>
<tr>
<td>Arginine</td>
<td>12,500</td>
<td>6,000</td>
<td>1,200</td>
</tr>
<tr>
<td>Tryptophane(^)</td>
<td>17,000</td>
<td>20,000</td>
<td>19,500</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>36,000</td>
<td>33,000</td>
<td>8,500</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>44,000</td>
<td>16,000</td>
<td>9,600</td>
</tr>
</tbody>
</table>

In the case of paramyosin, the hyperchromic increase on heating is partly reversible and there are no large changes in the shape of the band. The apparent red shift in the spectrum of this protein at the higher temperature (Fig. 6) is at least partly due to the temperature-induced broadening of the water absorption band. There is no indication of the shoulder at 205 mμ observed in the simple helical polypeptides, even though paramyosin is almost wholly helical. It is presumably masked by the superimposed side chain absorption.

Fig. 6.—Far ultraviolet absorption spectrum of (L) native and oxidized ribonuclease and (R) paramyosin, showing increase in absorbance on heating. Markings show peak absorbance expected for disordered form and helical form, taking into account side chain absorption.

Three different wavelengths, 190, 197, and 205 mμ, were chosen for estimating the helical contents for the various proteins. Average molar extinction coefficients for the peptide groups were used, based on the spectra of poly-L-lysine and poly-L-glutamic acid, corrected for carboxylate absorption in the random form.
Fig. 7.—Short wavelength ultraviolet absorption spectrum of insulin.

These average values are given in Table 2. The largest fractional change occurs at 197 μ.

**TABLE 2**

**Residue Extinction Coefficients as a Function of Conformation**

<table>
<thead>
<tr>
<th>Protein</th>
<th>190 μμ</th>
<th>197 μμ</th>
<th>205 μμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random coil</td>
<td>6,900</td>
<td>6,350</td>
<td>3,200</td>
</tr>
<tr>
<td>α-Helix</td>
<td>4,100</td>
<td>3,200</td>
<td>2,000</td>
</tr>
</tbody>
</table>

**TABLE 3**

**Comparison of Estimated Helical Contents of Several Proteins by Ultraviolet Absorption and Rotatory Dispersion Methods**

<table>
<thead>
<tr>
<th>Protein</th>
<th>190 μμ</th>
<th>197 μμ</th>
<th>205 μμ</th>
<th>Average</th>
<th>Rotatory dispersion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paramyosin</td>
<td>105</td>
<td>95</td>
<td>102</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>99</td>
<td>67</td>
<td>81</td>
<td>82</td>
<td>74</td>
</tr>
<tr>
<td>Insulin</td>
<td>96</td>
<td>44</td>
<td>57</td>
<td>66</td>
<td>&gt;51</td>
</tr>
<tr>
<td>Ribonuclease, oxidized</td>
<td>58</td>
<td>35</td>
<td>27</td>
<td>40</td>
<td>&gt;17</td>
</tr>
<tr>
<td>Ribonuclease, oxidized</td>
<td>18</td>
<td>18</td>
<td>19</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>β-Lactoglobulin, pH 6.4</td>
<td>33</td>
<td>25</td>
<td>31</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>β-Lactoglobulin, pH 8.7</td>
<td>23</td>
<td>14</td>
<td>12</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

The comparison is set forth in Table 3 for the few cases thus far available. The estimated helical contents from rotatory dispersion are based upon b values and a standard value of −630 for 100 per cent helix, except in two cases. In the case of myoglobin, a somewhat different procedure was used in treating the optical rota-
tion data which was largely in the ultraviolet;\textsuperscript{22} in the case of oxidized ribonuclease, the estimate is based on $\lambda_c = 226.6$. The values given for insulin and ribonuclease are listed as minimal values because of the possibility that there are some left-handed helical regions in these molecules\textsuperscript{24} that cancel out an equivalent amount of right-handed helix. Such cancelation would not be reflected in the ultraviolet measurements. Finally, it should be pointed out that $\beta$-lactoglobulin at pH 6.4 has a very anomalous rotatory dispersion which has been interpreted as indicative of either very high or quite low helical content.\textsuperscript{22, 26}

In view of the limitations thus imposed, the comparison of the ultraviolet absorbance and rotatory dispersion values reveal a fairly good correlation, one that would appear to justify further work. Indeed, the variation of the estimates for a given protein at different wavelengths are the more disturbing feature of the comparison. The values at 190 m\textmu are often quite high. This may be due to the large contribution being made here by the aromatic residues and the strong dependence of this absorbance on wavelength near 190 m\textmu. Obviously, this requires further study. At present, it would appear that 197 m\textmu is the more reliable point of measurement, this being the point of greatest difference, as well as being displaced somewhat from the region of strongest benzenoid absorption. Indeed, the absorbance due to side chains is only about half as much at 197 as at 190 m\textmu. Insulin represents the most extreme case of the proteins measured: the side chain contribution per residue to the molar extinction coefficient is 6,990 at 190, 4,190 at 197, and 1,600 at 205 m\textmu. Thus, the choice of 197 m\textmu represents a useful compromise between shorter wavelengths where the side chain absorbance is proportionally greater and instrumental uncertainty more acute, and longer wavelengths where the absorbance itself is decreasing rapidly.

This tentative conclusion does not dismiss the possibility that the side chain absorption bands are in fact substantially modified when the chromophore is incorporated into the protein molecule, nor the possibility that other conformations with anomalous optical properties are present. It is hoped to answer these and other questions by investigating the effect of environment on side chain chromophores in the short wavelength region and observing the effect on the absorption band of conformational changes in a wide variety of proteins.

Summary.—The absorption spectra of polypeptides and proteins in aqueous solution in the region 185–240 m\textmu have been investigated, and the various factors contributing to this absorption have been analyzed. In polypeptides in the $\alpha$-helical form, a large hypochromic effect is observed, as well as a new feature in the form of a shoulder at 205 m\textmu, which is not present in the spectrum of the random coil conformation. The absorption spectrum associated with the $\beta$-form has also been recorded and is shown to differ from each of the other two. The hypochromic effect is also observed in proteins, and by applying a correction for the absorption of chromophoric side chains, it is possible to arrive at a value for the helical content of the protein, which agrees moderately well with data derived from optical rotatory dispersion measurements.

We have greatly benefited from discussion with W. B. Gratzer and M. Kasha throughout the course of this investigation. This work was supported by the Office of Naval Research (N5 ori-07654.)
POLARIZATION OF THE ULTRAVIOLET ABSORPTION BANDS IN 
α-HELICAL POLYPEPTIDES*

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An analysis by Moffitt\(^1\)\(^2\) of the optical properties of the peptide chromophore in an α-helical array indicated firstly that the principal transitions should be optically active, and secondly that the \(N \rightarrow V_1\) system of this chromophore\(^3\) would give rise, by resonance exciton interaction of the electronic transition moments of the oriented peptide groups, to two bands. These would take the form of a component polarized parallel to the helix axis and a degenerate pair with perpendicular polarization. Moffitt's calculations were based on spectroscopic data for simple