Optical Activity of Cystine-containing Proteins

II. CIRCULAR DICHROISM SPECTRA OF PANCREATIC RIBONUCLEASE A, RIBONUCLEASE S, AND RIBONUCLEASE S-PROTEIN*

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SUMMARY

The circular dichroism spectra of pancreatic ribonuclease A, RNase S, and RNase S-protein have been measured in the wave length interval 198 to 300 mμ under various conditions. The circular dichroism spectra of RNase A and S are very similar, possibly identical, over this entire wave length interval at neutral pH. That of RNase S-protein is substantially different. It is shown that the portion of the RNase A spectrum in the peptide-absorbing region may be fitted by assuming that 11.5% of the residues are in an α-helical conformation and 33% in a β-conformation. Two of the three characteristic bands of the α-helix (poly-α, L-glutamic acid) and both of the characteristic bands of the β-structure (poly-α-lysine) were not adjusted in any way. However, the shortest wave length α-helical band (192 mμ) and the random coil band at 198 mμ were allowed to vary, in intensity, for best fit. In addition, it was necessary to include a positive band at 226 mμ. Arguments justifying this inclusion are given and depend largely on the presence of a small band observed in native RNase A at or near 240 mμ. The latter is believed to result from overlap of the (assumed) positive band near 226 mμ and the much more intense negative bands due to peptide transitions. In RNase S-protein, the far ultraviolet bands are considerably diminished in intensity, but the maximum near 240 mμ is greatly intensified and shifted by 3 to 4 mμ to shorter wave lengths. It appears likely that the anomalous positive circular dichroism near 240 mμ is generated, or contributed to, by aromatic residues, some of which may be titratable tyrosines. However, titration, acetylation, and nitration results failed to establish this conclusively. A larger, negative, band at 275 mμ is shown, in agreement with others, to arise predominantly from tyrosine residues. In this case, also, modification experiments did not allow us to decide relative contributions to the band by exposed and masked tyrosine residues. There may also be a small component due to optically active disulfide transitions.

Circular dichroism bands generated by side chains of ribonuclease A were first reported about 4 years ago (1). Since that time, several groups of investigators have examined various aspects of the bands originating in peptide as well as side chain chromophores in the near and far ultraviolet regions of RNase A, RNase S, RNase S-protein, RNase S-peptide, and RNase S' (2–8). During this time interval, x-ray diffraction studies of ribonuclease culminated in the publication of the crystal structures of RNase A (9) and RNase S (10). In the same period, instruments for measuring circular dichroism, of wider range and of wider range, have become available. However, the interpretation of the CD spectra of RNase A and its derivatives remains complex and uncertain. The far ultraviolet, peptide-dominated region of the CD spectrum is difficult to assign securely because the characteristic α-helical bands are not apparent (7), because ribonuclease contains very appreciable amounts of β-structures (9, 10), and, perhaps, for other, less obvious, reasons. The near ultraviolet region of the spectrum, too, has resisted definitive interpretation. At neutral pH, two nonpeptide bands are observed in the wave length interval 235 to 320 mμ (1). The larger of these occurs at about 275 mμ and is negative in sign. Near 240 mμ, there is a smaller, positive band. The CD band at 275 mμ has been assigned by different investigators to accessible (4) and inaccessible (2) tyrosine residues. Apparently conflicting results of alkaline titration and acetylation with N-acetylimidazole led to these different views. Titration leads to alteration of intensity of the band and a red shift of 13 mμ as the pH is raised from 7 to 11.5. Acetylation of the 3 exposed tyrosine residues, however, has been reported to have little or no effect on the band. There is, at the moment, very little known about the optical activity of aromatic residues in proteins and, indeed, in simpler model compounds containing aromatic residues. The complexity of the spectra of these compounds in the near ultraviolet (3) makes definitive assignments...
very difficult, but the conformational dependence of the spectra strongly justifies their detailed study.

In this, and the accompanying paper (11), we present results of investigations of the CD spectra, in peptide- and non-peptide-absorbing regions of RNase A, RNase S, RNase S-protein, reduced RNase A, and reoxidized RNase A. In particular, attention is focused in this paper on resolution of the far ultraviolet spectra and on examination of the positive band at 240 μm. The following paper is concerned with differences in spectra of RNase A and of various reduced-reoxidized forms.

**EXPERIMENTAL PROCEDURE**

**RNase: Preparation of Solutions and Enzyme Assay—**Bovine pancreatic ribonuclease A, lyophilized, phosphate-free, Lots 6089-90, 6508, and 6 JB, was purchased from Worthington. Although the optical properties of all three preparations were identical, Lot 6508 possessed 10 to 15% greater activity. The fractional activities of modified proteins, when cited, refer to the specific preparation of native enzyme used in an experiment. Enzymatic assays were performed by the Kunitz method (12) with yeast ribonuclease (reagent grade, Nutritional Biochemicals) as the substrate. The optical density at 300 μm was recorded on a Cary model 14 recording spectrophotometer. A Thomas circulating bath was used to thermostate the sample compartment. Concentrations were determined spectrophotometrically on the Cary or on a Beckman model DU spectrophotometer equipped with a Gilford model 220 read-out. Between pH 5 and 9, an extinction coefficient of 9.8 × 10¹ at 277.5 μm was sometimes used, but generally concentrations were measured at an isosbestic point, 280 μm, with molar extinction coefficients of 9.14 × 10¹ for native and 8.55 × 10¹ for reduced RNase. When necessary, scatter corrections were applied (14).

**Reagents—**Urea used in the early reduction studies, and when reoxidation was carried out in the presence of 8 M urea, was recrystallized from 50% aqueous ethanol by the method of Levy and Magoulas (15). In later work, the reagent was freed of cyanate by acidification to pH 2.0 for at least 50 min (16) and stored in this fashion. No effects could be discerned as a result of this change in procedure.

2-Mercaptoethanol was obtained from Eastman and used without purification. Nitrogen, extra dry, 99.7% was obtained from Matheson. The sodium salt of iodoacetic acid, used to carboxymethylate reduced ribonuclease, was obtained from Calbiochem. N-Acetylthiurea, purchased from K and K Laboratories, was desiccated under reduced pressure over P₂O₅ prior to use. Hydroxylamine hydrochloride, analytical grade, was obtained from Mallinkrodt. Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) and tetranirotomethane were obtained from Aldrich. 2-Methyl-2,4-pentanediol, used in the crystallization of both native and reduced reoxidized RNase, was purchased from Eastman. All other reagents were Fisher certified.

**Column Chromatography—**Sephadex G-25 (coarse) and G-75 were obtained from Pharmacia, and Amberlite CG-50 (replacing IRC-50), 200 to 400 mesh, chromatographic grade, from Mallinkrodt. The latter was prepared for use as described by Flis, Moore, and Stein (17). Chromatography columns were of the standard type except that a burette was used for those of 1-cm diameter. The void volumes of Sephadex columns were determined with blue dextran. Fraction collection was generally manual, although a Beckman model 132 fraction collector equipped with a model 133 refrigeration unit was used during the preparation of RNase S. The flow was monitored either by a Gilson ultraviolet lamp connected to a Texas Instrument recorder or by an ISCO model UA ultraviolet analyzer.

**Circular Dichroism Measurements—**CD measurements were performed on a Jouan Dicrographe modified for 10-fold greater sensitivity (18) or on a Cary model 60 spectropolarimeter with CD attachment. Molecular ellipticity values, in degrees square centimeter per decimole, are presented on a mean residue weight basis. All measurements recorded at wave lengths shorter than 200 μm were made on the Cary instrument. Both instruments agreed over the entire mutually accessible spectral range except for a difference, elaborated on under "Results," in the intensity of a band at 240 μm. At neutral pH and room temperature, the signal to noise ratios for this band on the Jouan often did not exceed 3. On the Cary, the signal to noise ratio is better in this region of high absorption and low dichroic intensity. The Cary base line, on the other hand, drifts noticeably at very high sensitivity. Under conditions of greater intensity of the band (low temperature, alkaline pH, or protein modification) there was no discrepancy between results with the two instruments.

**Preparation of Urea Solutions—**Urea solutions of the enzyme were prepared by bringing 0.8% volume of 10 M urea at pH 2 to the desired pH and then adding an aliquot of an RNase stock solution. Final adjustments of pH and volume were then made. Phosphate was generally added to the concentrated urea solutions in the solid form. The pH was then adjusted and the protein was added in the usual fashion.

**O-Acetylation of Ribonuclease—**O-Acetylation was performed as described by Rövden, Wacker, and Vallee (19), with a molar ratio of reagent to protein of 180:1. The reagent was dried under reduced pressure prior to use.

After standing for 1 hour at room temperature, the entire solution was placed on a Sephadex G-25 column (1 × 31 cm) and eluted with distilled water. Two-milliliter fractions were collected at a flow rate of 1 ml per min. The optical density at 280 μm was recorded and the concentration was determined with 5.66 × 10¹ (2) as the molar extinction coefficient.

The reaction was also conducted in the presence of 5 M urea. An aliquot of a ribonuclease stock solution was added to 4 ml of 10 M urea which had been brought from pH 2 to 7.4. The volume was adjusted to 5 ml. N-Acetylthiurea (a 180-fold molar excess), 24.9 mg, was added and the solution was allowed to stir for 1 hour at room temperature before gel filtration on Sephadex G-25. The column, 1 × 30 cm, was eluted with 5 M urea, pH 7. The flow rate was very slow and collection was interrupted overnight. Fractions, 2 ml, were collected. Concentrations were determined spectrophotometrically with an extinction coefficient of 3.18 × 10¹ at 260 μm (19). When desired, the acetyl groups were removed by the addition of hydroxylamine. An aliquot of the acetylated protein was combined with sufficient hydroxylamine hydrochloride (1 M in 8 M urea, pH 7.3) so that the final concentration of the deacylating reagent was 0.1 M. The pH was adjusted to 7.3, and the volume to 2 ml. The solution was allowed to stand for 2 hours at room temperature before recording the absorption and circular dichroism spectra.

**Reaction with Tetranirotomethane—**Ribonuclease, 8.2 mg, was dissolved in 2 ml of 0.05 M Tris buffer, pH 8.01. Tetranirotomethane, 0.1 cc of a 100-fold dilution, in 95% ethanol was added. The solution was allowed to stand overnight at room temperature.
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and then placed on a Sephadex G-25 column, 1.7 X 30 cm. Two-
milliliter fractions were collected with 0.1 m KCl as the eluent.
The concentration was determined with 6.60 X 10^3 as the
extinction coefficient at 381 myi. (This assumes reaction of
three tyrosines (21).)

Subtilisin Digestion of Ribonuclease—The subtilisin digestion of
RNase A was performed by the method of Richards and Vithayathil (22). RNase A, 336 mg, was dissolved in 2 ml of 0.1 m KCl
and transferred to the cell of a Radiometer TTTlc pH-stat by
means of an additional 0.3 ml of solvent. The pH was adjusted
to 8.0 and 0.092 ml of a freshly prepared 0.5% aqueous solution
of subtilisin (Subtilopeptidase A, bacterial, type VII, crystallized
and lyophilized, Lot 64B-1470, Sigma) was added. The pH was
maintained at 8.0 by continuous addition of 0.1 n NaOH and
digestion was continued for 3 hours at 0-2°. It was then stopped
by the addition of 1 n HCl, bringing the pH to 3.

Ribonuclease S was isolated from the other components of the
reaction mixture by ion exchange chromatography on an Amber-
lite CG-50, 200 to 400 mesh, column, 4.75 X 45 cm. The
eluent was 0.2 m phosphate buffer, pH 6.42 (17). A Beckman
fraction collector was used to collect 15-ml fractions. The
effluent was monitored at 280 mm by a Gilson ultraviolet flow
monitor and a Texas Instrument recorder. Two peaks ap-
peared. The second was designated RNase S because it was
enzymatically inactive in the presence of trypsin.

For further study, the fractions of the RNase S peak were
pooled, desalted by dialysis, and lyophilized.

RNase S-protein and RNase S-peptide were prepared by
trichloracetic acid precipitation as described by Richards and
Vithayathil (22). The trichloracetic acid precipitate, comprising the S-protein
fraction, was redissolved in 2 ml of water and purified by gel
filtration on Sephadex G-25. Distilled water was the eluent.
Lyophilization of the trichloracetic acid-free, aqueous layer, after ether extraction of the supernatant, yielded the S-peptide
in the form of a powder, which had a slight yellow-orange color.

RNase S-peptide, Lot 37B-8500, and S-protein, Lot 37B-8510,
were also obtained commercially from Sigma. Both were Grade
XII-PR and prepared from bovine pancreatic RNase S, type
XII-S, by trichloracetic acid precipitation. The commercial
peptide was not colored.

RNase S' was prepared by the addition of an equivalent of
S-peptide to a solution of RNase S-protein.

RESULTS

Far Ultraviolet Region—Fig. 1 shows the CD spectra of RNase
A, RNase S, and RNase S-protein in the spectral region 240 to
195 myi. The spectrum of RNase A shown here differs only very
slightly from that recently reported by Schellman and Lowe (7)
in that the extremum at 210 my appears to be a little less intense
and there may be a difference of 1 or 2 my in the position of this
maximum. In all other respects, the agreement is very good.

Not very noticeable in the case of RNase A, but important, is
the fact that between 236 and 238 my the CD becomes positive;

![](image)

**Fig. 1.** Circular dichroic spectra of RNase A, RNase S, and RNase S-protein at 25° and 3°. Spectra were recorded on Cary model
60 spectropolarimeter.
this is easily discerned in RNase S-protein in which the effect is encountered at shorter wave length.

At 25°, the spectra of RNase A and RNase S are virtually identical between 240 and 204 μm; lowering the temperature to 3° has no effect whatever, at this pH, on the spectrum of RNase A; there is a slight change at 216 μm in RNase S, but this may be within the experimental error. RNase S-protein, at 25° and at 3°, exhibits spectra similar in shape to those of RNase S and RNase A, but differing substantially in intensity. Sherwood and Potts have shown, by difference spectral studies, that partial normalization of inaccessible tyrosine residues in S-protein occurs at 25° (their conditions were pH 6.8, 0.01 M phosphate buffer), but their data indicate that at 3° the temperature-dependent transition has not begun (23).

It is possible to construct a curve which superimposes the experimental spectrum of RNase A (and S) everywhere in the wave length interval 196 to 236 μm by assuming that the composite spectrum comprises several bands, as given in Table I. The fit was arrived at visually with a du Pont 310 curve resolver.

To begin with, we assumed that the features of secondary structure observed in the crystal are preserved in solution, and that the characteristic bands generated by the α-helix and random coil structures of poly-α-L-glutamic acid (24, 25) and the β-structure of poly-L-lysine (26-28) would satisfactorily represent the observed far ultraviolet spectral bands. The half-band widths, wave lengths of maximum ellipticities, and maximum amplitudes for the α-helix, random coil, and β-structures are

<table>
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<th>Band</th>
<th>α-Helix</th>
<th>β-Structure</th>
<th>Random Coil</th>
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<td>RNase A</td>
<td>Fraction</td>
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<tr>
<td>λmax</td>
<td>ωmax</td>
<td>Δα</td>
<td>λmax</td>
</tr>
<tr>
<td>1</td>
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<td>4000</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>198</td>
<td>-30,700</td>
<td>10</td>
</tr>
</tbody>
</table>

* Δ is the wave length interval, in millimicrons, over which the ellipticity decreases from [ο]max to a value of [ο]max/є.

1 Intensity adjusted for best fit. Correct value for [ο]198 would be 7700.

2 See text: band not intended to correspond to Band 2 of random coil.

3 Intensity adjusted for best fit. [ο]198 = -17,170 would account for 100% secondary structure.

4 Values represent 11.5% α-helix based on the helical bands at 222 and 207 μm; 33% β-structure based on the bands at 217.5 and 195 μm; and 39% random coil based on the band at 196.5 μm.

There are several satisfying and several discomfitting aspects to this resolution. The satisfying aspects are that the characteristic gaussian parameters for four of the six bands were not adjusted in any way and the secondary structures arrived at in this way are in very good agreement with the known crystalline structure. The two bands that required adjustment were the 192 μm band of the α-helix and the 198.5 μm band of the random coil. For the former, the half-band width used was that of the resolved band in poly-α-L-glutamic acid but the intensity is only 3,400 deg cm² per decimole instead of 7,700 deg cm² per decimole, the value to be expected for a molecule containing 11.5% of residues in α-helical segments. In addition, the wave length of maximum amplitude is 2 to 3 μm longer than that observed in the synthetic polypeptide. For the random coil band, both its position and band width are those observed with poly-α-L-glutamic acid but its intensity is only 12,000 rather than 17,170. It is for this reason that the secondary structures add up to less than 100%.

Finally, it should be noted that a small positive band has been included at 226 μm. We comment below on the justification and significance of inclusion of this band as well as on possible reasons for the several approximations discussed above.

Near Ultraviolet Region—Fig. 2 shows the near ultraviolet CD spectra of RNase A at neutral pH and at several alkaline values. Denaturation with alkali abolishes the negative band altogether. It will be observed that both bands are altered as the pH is raised, in agreement with reports of Simpson and Vallee (2) and Simmons and Glazer (4). At neutrality and in weak acid, at 25°, the small positive band at 240 μm is difficult to measure reliably, although it is always apparent. An average value for the band is 80 deg cm² per decimole, but the range is from about 50 to about 100, depending on the instrument used and the
preparation. The Cary gave a consistently higher value than the Jouan. In any event, it is questionable whether the precision is better than ±20% for this band with either instrument with a single preparation. The precision of measurement is greatly improved when the band is more intense, as results from elevating pH, lowering temperature, or modifying with subtilisin (see below). The negative band near 275 μm is readily measured with a precision of ±5%. It is worthwhile to recall here that the overlap of two gaussian bands of opposite sign results in an apparent separation of band maxima greater than the actual separation of the isolated bands, as well as in a diminution of intensity of each (20). If the two bands are of different intensity, then the smaller of the two suffers greater displacement from its isolated position. In the present case, there is evidence (see below) that the 240 μm band is only slightly overlapped with the 275 μm band, but strongly overlapped with the intense negative band system at shorter wavelengths (Fig. 1).

When the temperature is dropped below 5°, the maximum amplitude of the positive band increases 2.5- to 3-fold to an average of about 200 deg cm⁻² per decimole. There is no change in the intensity of the negative band at 275 μm and no discernible change in the far ultraviolet bands shown in Fig. 1.

**RNase S and RNase S-protein**—Fig. 3 (A and B) shows the near ultraviolet CD spectra of RNase S and S-protein. In the case of RNase S at both 25° and 4°, the 275 μm band is the same as that of RNase A. The 240 μm band is less intense than the average for RNase A, but just inside the lower limit of experimental uncertainty. At low temperature, the peak is 20% smaller than in RNase A. RNase S-protein shows a weaker band at 275 μm, but a greatly intensified band near 240 μm, the actual position being 237 and 236 μm at 25 and 6°, respectively.

Simons and Blout (5) have previously reported the absence of this band in RNase S-protein. However, more recently, these authors have observed this band in CD studies of RNase S-protein. However, more recently, these authors have observed this band at low temperatures in both phosphate and sulfate at pH 6.5 (29).

**Reaction of RNase with N-Acetylimidazole**—The near ultraviolet CD spectrum of RNase in which 3 tyrosine residues (as well as 7.5 to 8 amino groups) are acetylated (19) is shown in Fig. 4. The far ultraviolet band (not shown) shows no change to 215 μm and the negative band at 275 μm is not affected in position and only very slightly altered in intensity. The latter observation agrees with that of Simpson and Vallee (2). However, the 240 μm region of the spectrum, which was not previously examined in CD studies, shows a marked increase in ellipticity.

Vallee and his co-workers have shown that, under the conditions which were used in this study, accessible tyrosines in proteins undergo reaction to form O-acetyltyrosine residues. The spectral maximum normally observed at 275 μm is then blue-shifted to 263 μm and there is a decrease in molar extinction at 280 μm of 1160 for each mole reacted (18). In agreement, also, with the findings of Simpson and Vallee (2), we find the enzyme to be about 85% active under these conditions.

If the reaction is carried out in the presence of 8 M urea, all 6 tyrosine residues of ribonuclease react, yielding an enzymatically inactive derivative (2). The characteristic absorption band at 277.5 μm is shifted to 263 μm.

The CD spectrum of completely acetylated RNase in 8 M urea is shown in Fig. 5, which also shows the results of deacetylation in urea. The 240 μm band is altogether absent while the band at 275 μm is blue-shifted and sharply reduced in intensity.

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**Fig. 3. A,** circular dichroic spectra of RNase S-protein in the region of side chain absorption. Measurements were made on a Cary model 60 spectropolarimeter. **B,** circular dichroic spectra of RNase S in the region of side chain absorption. Measurements were made on a Cary model 60 spectropolarimeter.
Removal of the acetyl groups (with hydroxylamine) restores the absorption spectral band to 275 μm, but not the CD maximum, although some of the intensity of the CD band is regained. This spectrum is not identical with that of RNase in 8 M urea (see below). If the deacylation is carried out after removal of urea, much of the CD spectrum of native RNase is regained, with the major difference being a band at 275 μm smaller than that observed with the native protein. The enzymatic activity is restored to 50 to 65% of its original value (2).

CD Spectrum in 8 M Urea: Effect of Phosphate Ion—Optical rotatory dispersion studies by Sela and Anfinsen revealed that the three-dimensional structure of ribonuclease was considerably disrupted in the presence of 8 M urea (30). These authors found, also, that the addition of 0.15 M phosphate to solutions of RNase in 8 M urea resulted in virtually complete restoration of the optical rotation of native RNase. It is known that RNase is fully active against RNA in 8 M urea (31). Sela and Anfinsen suggested that this was due to the polyphosphate function of RNA and this has been borne out by all investigations.

The findings of Sela and Anfinsen were based largely on analysis of the optical rotation at the sodium D line which is primarily influenced by large far ultraviolet bands, arising mainly from peptide optical activity of RNase. The far ultraviolet CD band which we observe is, indeed, drastically reduced. The near ultraviolet bands (Fig. 6) (which contribute only negligibly to the optical rotatory dispersion in the visible) are also markedly affected. Again, the 240 μm band is absent and the 275 μm band, while unaltered in position, is reduced by about 40% at pH 8. If the pH is raised to 11.0 (all six tyrosines are titratable in 8 M urea (32) and the apparent pK is about 10.7) the intensity of the band is further reduced, now to about 10% of the value of native RNase, but there is no indication of a shift to longer wavelength.

Addition of 0.15 M phosphate has a pronounced effect on the near ultraviolet CD spectra in 8 M urea. In Fig. 7 are shown CD spectra in 8 M urea with phosphate added at different pH values. In acid, phosphate has no effect. In fact, acidification results in a blue-shifted band which is much smaller than the band at neutral pH in the absence of phosphate. As the pH is
increased and the proportion of dibasic phosphate consequently increased, the CD spectrum begins to resemble that of native RNase in the absence of urea. When half of the phosphate is dibasic, the positive dichroism at 240 reoccurs and the negative band achieves about 80% of its normal value. The ellipticity at 220 μm increases to about 85% of that of native RNase after having fallen to 10% in acid. These are not ionic strength effects, since variation of added KCl to RNase from 0 to 1 M has no effect on the CD spectrum, but other anions may give the same result. We have tested only phosphate.

**DISCUSSION**

**Analysis of Far Ultraviolet Region**—As noted under "Results," the fit which we have presented for the far ultraviolet ellipticity bands of RNase A has several disquieting features. The resolution is presented to indicate that it may not be necessary to invoke markedly altered optical parameters due to short or distorted helices. There appears to be little doubt that it is necessary to include at least one positive band centered between 225 and 236 μm. In Table I, this band is given an intensity of 1,500 deg cm² per decimole at 226 μm. It is more likely that correct resolution would yield a shorter wave length band (perhaps the 217 μm random coil band) and a slightly longer wave length band arising from side chain contributions. It did not seem to us worthwhile to do this additional resolution because we would add an additional adjustable parameter for entirely *ad hoc* reasons. However, there is simply too much positive intensity at 239 μm in the native protein at 3° and at 236 μm in S-protein to allow a resolution which overlooks this band. Schellman and Lowe (7) have noted, quite correctly, that the far ultraviolet CD spectrum exhibits insufficient negative dichroism over the interval 220 to 235 μm (approximately) for any reasonable amount of α-helix displaying a poly-α, L-glutamic acid type n - π* Cotton effect. Their way of accounting for this involved a displacement of the n - π* Cotton effect by about 5 μm to shorter wave lengths. The result was a very good fit to the experimental curve from 200 μm to about 230 μm, but at longer wave lengths the experimental points were more positive than the calculated ones, reflecting the anomalous positive dichroism of RNase near 240 μm. Schellman and Lowe argued from two kinds of evidence that side-chain positive ellipticity generated by aromatic side chains could not account for the anomalous blue-shifted CD spectrum: (a) extremely large rotatory strengths would be required of the six tyrosines and (b) titration to pH 11.3 has no effect on the Cotton effect at 217 μm. Both of these points are important and not easily painted over. The first is true, however, only if the positive contributions resulted from less than the majority of aromatic residues present. In Fig. 8 are shown far ultraviolet CD bands of N-acetyltirosineamide at pH 7 and pH 12 and of N,O-diacyetyltyrosineamide at pH 7.3. Since tyrosine residues comprise about one-twentieth of the total molecule, a contribution equal to that of the model compound could generate most of the intensity required by our fit. In addition, the 3 phenylalanine residues could account for 250 deg cm² per decimole on a residue basis. The second line of evidence cited by Schellman and Lowe is quite compelling. However, examination of Fig. 8 reveals that ionization of 3 residues would lead to a maximum change of less than 200 deg cm² per decimole between 230 and 215 μm, and this would be difficult to detect securely. At longer wave lengths, the CD spectra are different in the two ionization states.

Both arguments favoring the fit that we have used stretch credibility somewhat, but they are not necessarily more drastic than the substantial change in excitation energy required for the n - π* transition to be displaced by 5 μm. It may be that alteration of characteristic helical parameters as well as side chain...
influences combine to produce the anomalous CD spectrum of RNase A.

The second difficulty with the fit presented in Table I is the need to use a lower value for the large random coil band at 198 m\textmu than is observed with either disordered poly-\(\alpha\)-L-glutamic acid or poly-L-lysine. Thus, the maximum intensity used for the random coil band is only about 70% of the value found for the polymers. Although, there is still some disagreement among laboratories as to the precise value to be used, the discrepancies are not as great as 30%. A possible justification for the procedure, although hardly convincing, is that ribonuclease which has been reoxidized in urea or ascorbic acid also yields a value at 198 m\textmu only about two-thirds of that exhibited by the disordered polymers.

These comments are made without any attempt to minimize the hazards of curve fitting. It would be grossly misleading to suggest that a unique fit has occurred, or is possible in this instance. Many more proteins, of known structure, will have to be examined before a high level of confidence can be attached to such procedures.

The main points to stress, however, are:

1. The possibility of fitting the data without necessarily altering parameters for \(\alpha\)-helical and poly-L-lysine-like \(\beta\)-conformations.

2. The very sensitive dependence of dichroism near 240 m\textmu on slight (possibly nondiscerned) changes in dichroism at shorter wave lengths.

3. The certainty of side chain contributions at wave lengths between 225 and 240 m\textmu.

Near Ultraviolet Region—The band near 240 m\textmu is affected by modifications involving accessible tyrosine residues. Examination of Fig. 2 reveals substantial intensification and wave length alteration between pH 8.9 and 11. This finding, of itself, is not, however, definitive evidence that tyrosine transitions generate the band at neutral pH, since it is possible that residues are optically active only in the ionized state. The tentative assignment to accessible tyrosines is, however, further justified by the increase in intensity at 240 m\textmu resulting from acetylation of 3 tyrosine residues. Moreover, nitrination with tetranitromethane results in diminished intensity at 240 m\textmu and in decrease in the pH at which ionization of accessible tyrosine residues occurs, in accord with the expected lowering of the pK of phenolic ionization resulting from ortho-nitrination (Fig. 9).

From these results, as well as from the effects of urea (Figs. 6 and 7) and of reversible acid denaturation (3), it would appear that the small positive band near 240 m\textmu is due at least in part to transitions at shorter wave lengths of 1 accessible tyrosine residue or more, the optical activity of which depends on the integrity of the native structure. Simons and Blout, from somewhat different lines of evidence, have made a similar assignment (5).

The difficulty in secure assignment, in our view, arises from the overlap of this band with the much more intense bands at shorter wave lengths. Thus, minor alterations in the intensity of the CD at 220 m\textmu would lead to profound alterations in the relatively weak band observed near 240 m\textmu. In RNase S-protein, for example, the extraordinary amplitude of this band (shifted to 237 m\textmu) may result primarily or entirely from diminished negative contributions due to peptide transitions, rather than from profound alteration in the environment of 1 aromatic residue or more. Much smaller disturbances related to peptide optical activity could account for the effects produced by N-acetyl-L-tyrosine ethyl ester and tetranitromethane, and lowering of temperature to 5\(^{\circ}\) or less.

Finally, small though the band appears, arguments have been presented above suggesting that the band is actually the tail end of a larger gaussian centered at shorter wave lengths. If this is so, then residues other than the accessible tyrosines may contribute to the observed intensity, and changes in optical activity of the accessible residues would yield changes at 240 m\textmu diluted by the constant background arising from unaltered residues.

The CD band at 275 m\textmu is easier to observe and has been more extensively studied than the shorter wave length band at 240 m\textmu. The complex nature of the band is implied in its behavior as a function of pH. Tanford, Hauenstein, and Rands (33) showed that the pK of the normal tyrosines in ribonuclease was 9.9. Thus at pH 11, 90% of these residues are present in the phenolate form. If these were the residues responsible for the optical activity at 275 m\textmu, one would expect to see the band shift to 295 m\textmu in alkali. Likewise, there would be no change in its properties if the band reflected transitions of cystine or inaccessible tyrosine moieties, since these would be unaffected by the change in pH. Neither alternative is quite adequate to describe the data. The band undergoes a red shift to 288 m\textmu and shows a 50% increase in ellipticity at its maximum. This behavior is not typical of a normally titrating tyrosine, although such a chromophore may actually contribute to the optical activity in this spectral region.

Although it had previously been observed that the 275 m\textmu band moved as a function of pH, Simpson and Vallee (2) concluded that it could not be assigned to an exposed tyrosine because of the O-acetylation results. Instead, they postulated that one of the exposed tyrosines, which does not contribute to the band at neutral pH, becomes optically active in the phenolate form. Such an explanation accounts for most of the data but is contradicted by the results of reaction with tetranitromethane.

Fig. 9 shows a sharp decrease in optical activity in the 275 m\textmu region of the spectrum accompanies nitration. The reaction is also marked by the generation of several new circular dichroism bands in the spectral region above 300 m\textmu. These can be assigned only to nitrotyrosine. Above pH 6, a positive band appears in the vicinity of 290 m\textmu, while the 275 m\textmu band is further diminished. (Thus, at least in this case, the 250 to 300 m\textmu region of the spectrum comprises more than one transition). It seems plausible to assign the 290 m\textmu band to a nitrated tyrosine residue present in the phenolate form, while that at 275 m\textmu may represent either a buried tyrosine or a disulfide transition or both.

Since there is only a 20 to 25% decrease of ellipticity in the far ultraviolet and the modified enzyme retains 83% specific activity, it is unlikely that the protein has sustained a widespread conformational change. One is forced to conclude that the reactive tyrosines do contribute to the optical activity in the 250 to 300 m\textmu region of the spectrum of nitrated RNase, and most likely also to that of the native enzyme. Although this contribution appears negative in native ribonuclease and positive in the pH 6 spectra of nitrated RNase, an inversion of sign is not surprising. Both N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine amide behave similarly upon titration of the phenolic hydroxyl group (3).

Considering the apparent disagreement of such an assignment with the O-acetylation data, it is important to remember that
only a component of the 275 μ band need stem from an accessible tyrosine to explain the above results. Other components may also be present. Studies in our laboratory indicate that the O-acetylation of N-acetyl-L-tyrosine is accompanied in the CD by a 5 μ red shift and an approximate 40% decrease of intensity at the 270 μ maximum. The ellipticities at 265 μ are about the same in both molecules. Therefore, if half of the ellipticity at 275 μ were due to a reactive tyrosine chromophore, a 20% decrease would result on O-acetylation. We have observed a decrease of 10%; a smaller contribution from an exposed tyrosine would be difficult to discern. Thus the inclusion of such a component in the assignment of the 275 μ band is compatible with all of the observed data.

Studies of the effects of pH and O-acetylation on the RNase CD spectrum strongly suggest the presence of a component other than exposed tyrosine in the 275 μ band. Two possibilities exist for assignment of this other component: inaccessible tyrosine and disulfide.

The very presence of a band in 8 M urea is more characteristic of cystine than of tyrosine chromophores. Furthermore, it cannot be inferred that the same intramolecular interactions which render three tyrosines inaccessible in aqueous solution provide them with an asymmetrical environment in urea. Blumenfeld and Levy (32) have shown that the buried tyrosines are normalized in this solvent. In the presence of urea at pH 10.7, a small band appears at 275 μ. At this point, 50% of the residues are present in the phenolate form, and one would have expected to see a red shift.

Similarly, the near ultraviolet negative band of RNase S is not red-shifted at pH 11.

As this section was being written, Beaven and Gratzer (34) published a detailed study and analysis of the nitration of RNase. These authors studied spectral, rotatory dispersion, and circular dichroic properties of RNase at different levels of nitration. Their CD spectra, at lower levels of nitration than reported here, indicate that the appearance of a new unionized nitrotyrosine CD band near 260 μ is not accompanied by any diminution of the band arising from free tyrosine at 275 μ. This result is different from that reported here. On the basis of their results, Beaven and Gratzer conclude, in agreement with Simpson and Vallee, that the 275 μ Cotton effect in RNase is generated by masked tyrosines. We feel compelled to remain on neutral ground with respect to this assignment, largely because the protein has sustained a conformational change, albeit limited, at both low and high levels of nitration.

In summary, then, one may conclude that the 275 μ band comprises transitions of both tyrosine and cystine residues, the former dominating. In this, as in the case of insulin (1, 35), the possible effects of tyrosine and cystine alterations on one another are a complicating feature. Thus titration of a tyrosine residue located near a cystine may alter cystine optical activity, just as reduction of a cystine residue might (and probably does) affect optical activity of neighboring tyrosine residues.

We have not reported our results with RNase S' because of discrepancies between results with S-peptide prepared in this laboratory and that available commercially. The S-peptide prepared in this laboratory produces full enzymatic activity when combined with a stoichiometrically equivalent amount of S-protein. The S-peptide is, however, yellowish. Our results with this S-peptide are in accord with observations of Simons and Blout with regard to the absence of the small band near 240 μ. Other experiments with commercially available S-peptide required more than an equivalent for full activity and were, accordingly, rejected.

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