entirely explicable in terms of differences in amino acid content. We believe that the differences in binding which have been observed can in fact be reasonably explained in this way. The observed differences are actually very small. A theoretical discussion of the preferential hydration of native proteins has been presented by Shumaker and Cox,[3] and they show that several hundred solvent molecules come within the sphere of influence of the surface of a native protein molecule. The number of solvent molecules associated in close contact with a randomly coiled protein molecule must be considerably larger, *i.e.*, the number is likely to exceed 1000 for most of the proteins for which we have reported data. The preferential binding in terms of moles of GuHCl/mole of protein, which lies in the range of 0 to 53 (last column of Table IV), thus reflects very small preferences indeed, and individual differences of the kind observed would seem to be entirely reasonable, since the different amino acid side chains presumably differ from each other in their preference for contact with water or GuHCl.

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An Analysis of the Tyrosine Circular Dichroism Bands in Ribonuclease[1]

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Abstract: Ultraviolet circular dichroism curves have been measured for bovine pancreatic ribonuclease and for a model compound, N-acetyl-L-tyrosinamide, in neutral and alkaline solutions. The influence of ethylene glycol on the circular dichroism was also examined. The correspondence in the circular dichroism curves of ribonuclease and N-acetyl-L-tyrosinamide under various conditions is discussed in relation to the origin of the tyrosine Cotton effect exhibited by the native enzyme.

In a recent communication[2] we described near-ultraviolet Cotton effects in ribonuclease (RNase) which were sensitive to both pH and denaturing agents. The red shift and increase in amplitude of the Cotton effect in the region of the tyrosine absorption bands, which occurred on raising the pH from 6 to 11.5, led us to the conclusion that the readily ionizable tyrosine residues[3,4] made a major contribution to the observed anomalous dispersion. Recently, Simpson and Vallee[5] reported that reaction of native RNase with N-acetylimidazole led to a conversion of three tyrosine residues to the O-acetyl derivative. This reaction is associated with a large decrease in the absorbancy of tyrosine at 275 mμ and loss of optical activity associated with this tyrosine chromophore. Further, they found that whereas acetylation of native RNase led to the expected decrease in absorbancy at 278 mμ, there was *no concomitant change in optical activity at pH 6*. On the assumption that the modified residues were those showing normal ionization behavior, Simpson and Vallee[5] concluded that the major contribution to the anomalous dispersion arose from among the three "buried" residues. In view of these results, our explanation that the normally ionizing tyrosine residues contribute significantly to the total optical activity observed at neutral pH seemed no longer tenable. We have therefore undertaken an analysis of the circular dichroism (CD) of RNase in an attempt to resolve these apparently conflicting interpretations. The CD spectrum of a model compound N-acetyl-L-tyrosinamide (NACTA) was similarly examined.

Experimental Section

Crystalline bovine pancreatic ribonuclease, Lot R662-ML, was obtained from Worthington Biochemical Corp. All circular dichroism measurements were performed with a Jasco Model ORD/UV5 instrument. The data displayed in the figures are direct reproductions of the chart recordings and are therefore reported as $\Delta E = E_L - E_R$. The concentrations of RNAase and of the model compound were chosen so as to give the same molar concentration of tyrosine, and hence the magnitudes of the CD curves obtained may be compared directly

Results and Discussion

As can be seen in Figure 1, RNase shows a strong negative CD band centered at 273 mμ at pH 6, ascribable to tyrosine residues. On raising the pH to 11.5 there is a gradual shift of this band to a position centered at 285 mμ, that is, in the direction expected from the shift in the absorption spectrum associated with the ionization of tyrosine residues. The curves shown are similar to those recently reported by Beychok.[6]

If the CD band centered at 273 mμ is due to the "buried" residues and does not change in magnitude or

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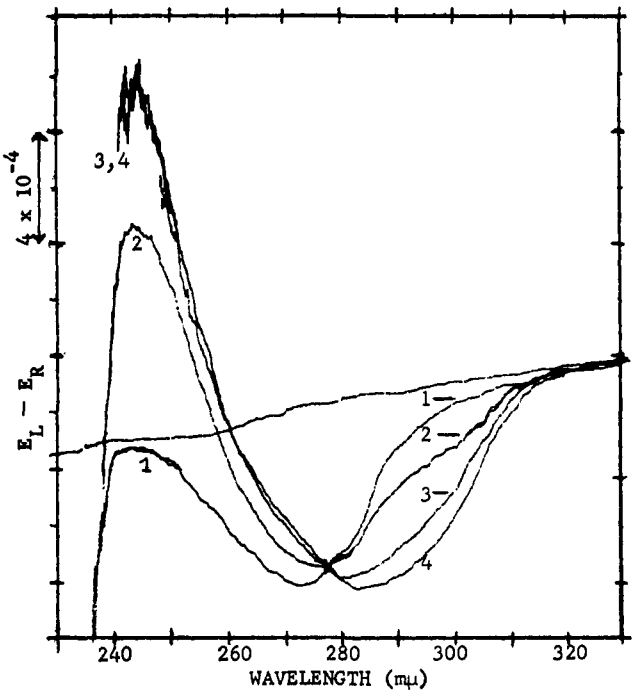

Figure 1. The circular dichroism of ribonuclease (2.8 mg/ml) as a function of pH: curve 1, H_2O; curve 2, in 0.2 *M* glycine–NaOH at pH 9.5; curve 3, at pH 10.5; curve 4, at pH 11.5; path length, 0.5 cm.

positive at 227 mμ, one negative at 270 mμ, and a smaller positive band at 286 mμ corresponding to the three bands in the tyrosine absorption spectrum. On raising the pH to 11.5, these bands all shift to the red by about 15 mμ, and it can be seen that at pH 11.5 the short wavelength positive band overlaps the position of the negative band at pH 6, with an isosbestic point at 277 mμ, and the rotatory strength of the CD bands remains similar at the two pH's.

It is striking that the effect of pH on the CD spectrum of NACTA is very similar to that observed with RNAase, and, indeed, the isosbestic point occurs at 277 mμ and again, in both cases, the rotatory strengths are similar at pH 6 and 11.5 (see Figures 1 and 2). There are two possible explanations of this observation. First, that it is indeed the readily ionizable residues which are exclusively involved. Second, to reconcile this observation with the interpretation of Simpson and Vallee,[5] it is necessary to conclude that the contribution of the "buried" residues decreases proportionately with the appearance of that due to the readily ionizable residues.

It is obvious that the interpretation of the CD in the critical region of 240–270 mμ is complicated by the overlapping contributions from at least three sources, *i.e.*, the positive and negative tyrosine bands, as well as a strong negative contribution from the polypeptide

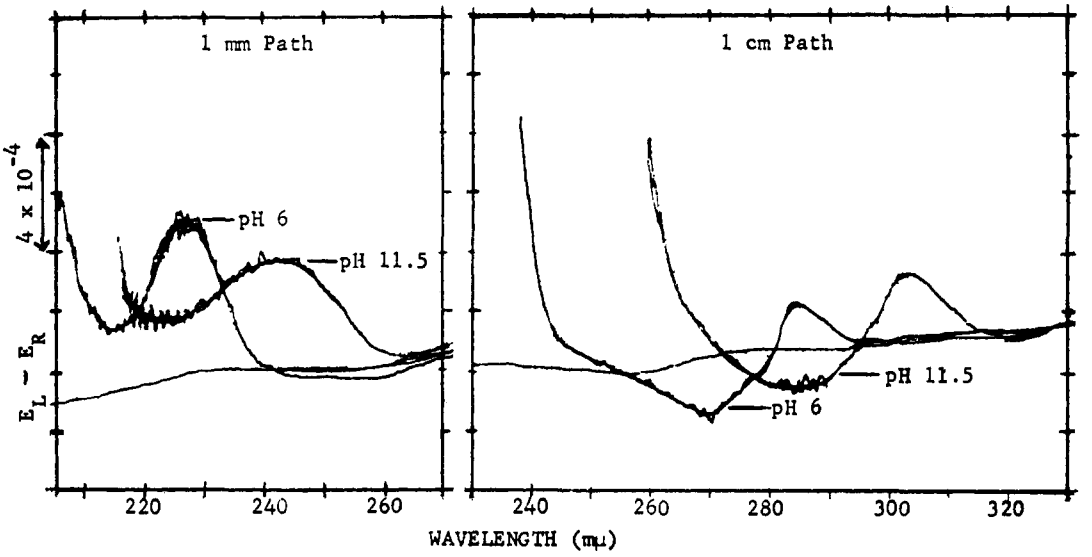

Figure 2. The circular dichroism of N-acetyl-L-tyrosinamide (1.5×10^{-3} *M*) at pH 6 in H_2O and at pH 11.5 in 0.2 *M* glycine–NaOH. Path lengths as indicated.

position with change in pH, one might expect broadening of the negative CD band at pH 11.5 so as to include the contribution of the red-shifted ionized residues, as might have been suggested by the magnitude and shape of the ORD spectra.[2,5] This is not the case. Thus, at first glance, it would seem that the ionizable groups are responsible for both the negative band observed at pH 6 and that seen at pH 11.5. However, at pH 11.5 a strong positive CD band appears which significantly overlaps the position of the negative band at pH 6, and it might be argued that it is obscuring the latter, were it still present.

Now, however, examination of the CD spectrum of NACTA at pH 6 (Figure 2) shows three bands: one backbone and one arising from the disulfide bonds.[6]

In the hope that a better separation of the various overlapping optically active transitions could be achieved, RNase and NACTA were examined in 80% (v/v) ethylene glycol (EG). As shown in Figure 3, EG shifted the near-ultraviolet CD bands in NACTA to the red. *Further, the negative band was enhanced and the minimum shifted to 273 mμ but with no corresponding increase in the magnitude of either of the positive CD bands.* Thus, the relative magnitudes of the various optically active tyrosine transitions are unequally affected by solvent polarity. EG at a concentration of 80% (v/v) enhanced the magnitude of the near-ultra-

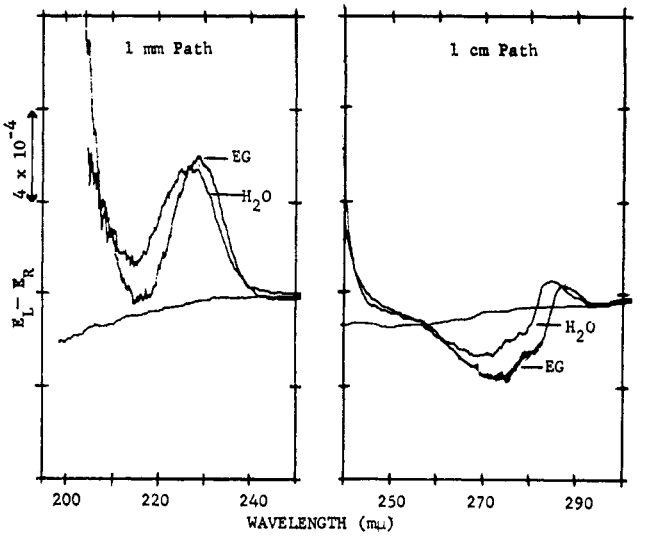

Figure 3. The circular dichroism of N-acetyl-L-tyrosinamide (1.5×10^{-3} *M*) at pH 6 in H_2O and in 80% (v/v) ethylene glycol (EG). Path lengths as indicated.

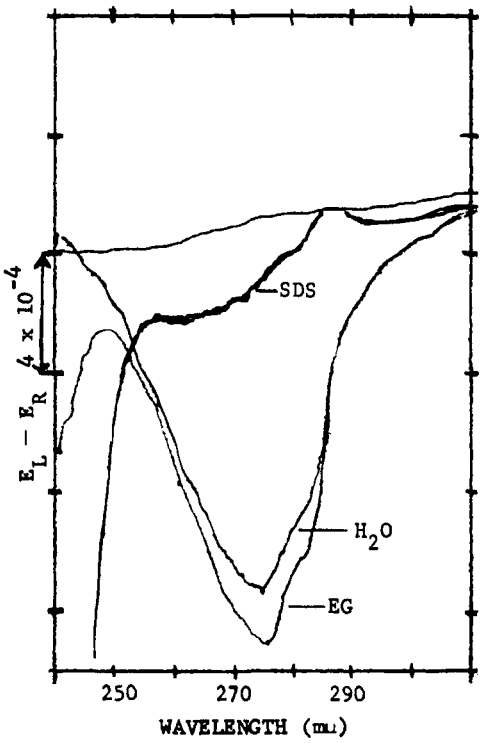

Figure 4. The circular dichroism of ribonuclease (2.8 mg/ml) at pH 6 in 2% sodium dodecyl sulfate (SDS), H_2O, and 80% (v/v) ethylene glycol (EG); path length, 1.0 cm.

violet CD band in RNase at pH 6 (Figure 4), in a manner similar to that observed with NACTA. This presumably represents the effect of the solvent on the exposed tyrosine residues. However, at pH 11.5, EG caused a marked diminution of the negative CD band (Figure 5). Thus, the orientation of the ionized tyrosine residues at pH 11.5 depends on interactions within the RNase molecule which are sensitive to EG. This is in sharp contrast to the situation seen at pH 6, where the CD indicates no disruption of the oriented optically active chromophores.

Exposure of RNase to sodium dodecyl sulfate results in the elimination of the negative CD band ascribable to oriented tyrosine residues (Figure 4), in

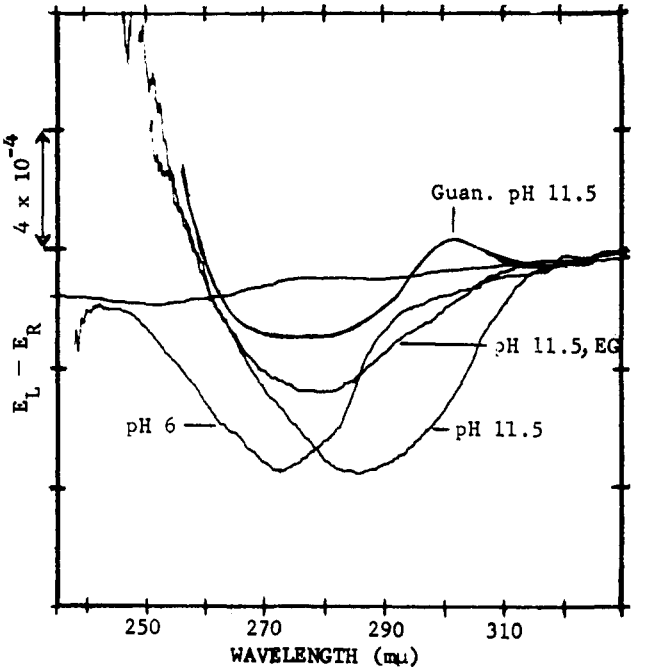

Figure 5. The circular dichroism of ribonuclease (2.8 mg/ml) at pH 6, pH 11.5, pH 11.5 in 80% ethylene glycol (EG), and in 5 *M* guanidine–HCl at pH 11.5. Path length was 0.5 cm for the first three, and 1.0 cm for the ribonuclease in guanidine. All solutions were 0.025 *M* in phosphate.

agreement with our previous ORD observations.[2] Since a similar profound disorienting effect was noted on RNase at extremes of pH,[2] it is apparent that ionic interactions are being disrupted in all cases, and the compelling conclusion must be drawn that electrostatic interactions play a significant role in the conformational stability of native RNase.

Despite the strong negative rotation (below 255 mμ) ascribable to the α helix, there can be seen a negative band or plateau which still persists in sodium dodecyl sulfate. Since this band also persists in 5 *M* guanidine hydrochloride, it is likely that it represents a contribution from the disulfide bonds[6] as well as the disoriented tyrosine residues. Finally, it should also be noted that NACTA shows a small positive CD band centered at 286 mμ which shifts to 304 mμ at pH 11.5. This band is not obvious in native ribonuclease, but is on exposure to sodium dodecyl sulfate, or guanidine (Figure 5). In these solvents, the near-ultraviolet CD spectrum of RNase resembles qualitatively that of the model compound NACTA in aqueous solution. It is noteworthy that the interactions responsible for the enhancement of the tyrosine CD in native ribonuclease do not increase the amplitude of the 286-mμ positive band as they do that of the 273-mμ negative band. This is also true for NACTA in ethylene glycol (*vide supra*).

The data presented here strongly suggest that the same residues are responsible for the tyrosine optical activity seen at neutral and alkaline pH. This interpretation rests on the similarity in the pH dependence of the rotatory strength and the identity of the 277-mμ isosbestic point in both RNase and NACTA. The data do not allow a decisive statement as to how much contribution the buried residues are making to the CD, if any. We are further studying the possibility that a normally ionizable tyrosine residue in RNase may not be susceptible to acetylation with acetylimidazole.