Optical Rotatory Dispersion and Circular Dichroism of Human Carbonic Anhydrases B and C*

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SUMMARY

Studies of optical rotatory dispersion (ORD) and circular dichroism (CD) are reported on human carbonic anhydrases B and C in the spectral region below 320 mp. Most of the observed CD spectrum of each enzyme could be described in terms of three principal Gaussian bands: a strong negative band near 216 mp, a weaker negative band near 270 to 275 mp, and a positive band intermediate between the other two in position. Above 260 mp, all CD values are negative; there is clear evidence of fine structure above 280 mp, involving at least two additional CD bands.

The ORD spectra above 260 mp show several peaks and troughs, which are characteristic for each enzyme. The principal troughs at shorter wave lengths lie at 222 mp for Enzyme B and at 226 mp for Enzyme C. At still shorter wave lengths, the ORD values rise to low peaks, at 204 mp, with [m'] = -300 for Enzyme B and +2750 for Enzyme C. These patterns are very different from those characteristic of either α-helical or β structures. Asymmetrical interactions involving the aromatic side chains almost certainly make important contributions to ORD and CD at the shorter wave lengths, as they clearly do at the longer wave lengths, above 250 mp.

On acid denaturation, the longer wave length Cotton effects vanish, and the ORD and CD spectra alter in such a way as to suggest the presence of 10 to 20% α-helix in the acid-denatured proteins.

The changes on exposure to high pH are more complex. Near pH 11, the negative CD band near 270 to 275 mp becomes less intense and shifts to longer wave lengths; the positive band at 232 mp in Enzyme B also shifts to longer wave lengths. It is tentatively suggested that these bands arise in part from interactions of tyrosine residues. The fine structure pattern between 280 and 310 mp undergoes changes, but in Enzyme B it still persists even at pH 13. Interactions involving tryptophan may be involved. The positive CD band at 249 mp in Enzyme C disappears at pH 11.5, and the ORD spectrum of this enzyme also indicates more drastic structural alterations at high pH than those found in Enzyme B. The ORD spectra of both enzymes at pH near 13 show two troughs, one near 231, the other near 210 mp, the former being deeper for Enzyme B, the latter for Enzyme C.

The CD bands of Enzyme B are still observed in 2 M guanidine-HCl, although the long wave ultraviolet bands are weaker than in the native protein; in 4 M guanidine-HCl they are abolished. ORD studies of the two enzymes in 1 M guanidine-HCl indicate that the Cotton effects due to the aromatic absorption bands disappear rapidly in this solvent in solutions of Enzyme C, whereas Enzyme B is much more stable.

ORD spectra have been calculated from the CD data for the native enzymes by the use of a Kronig-Kramers transform with a computer program. Comparison with the observed ORD data shows good general agreement with the pattern of the observed troughs and peaks, especially for Enzyme C. The computed spectra are displaced to more positive [m'] values than the observed spectra, indicating that there must be large negative contributions to the circular dichroism at wave lengths below 200 mp.

The rotatory dispersion of both human and bovine carbonic anhydrases shows unusual features (1-4), including a group of Cotton effects in the region between 260 and 300 mp. These are obviously associated with asymmetrical environments of the aromatic side chains, and they disappear on denaturation in acid, alkali, urea, or guanidinium chloride. The optical rotatory dispersion patterns at shorter wave lengths, between 240 and 195 mp, are notably different from those of proteins characterized by high helical content, and they do not closely resemble the rotatory dispersion spectra recently reported as charac-
teristic of the β structure (5-8). On acid denaturation, the dispersion patterns alter in a manner suggesting the presence of some helical content of the order of 20% (9). Here we report on the extension of the previous ORD measurements of Myers and Edsall (1) to shorter wave lengths and to high pH values. We have also studied in more detail the changes associated with denaturation in acid, at pH 4 and below, and have followed further the time course of the changes in ORD patterns under various denaturing conditions. We report circular dichroism data for these proteins, in the native and in several denatured states, with computations of the ORD patterns to be expected from the CD measurements, and comparisons between these and the observed ORD spectra. Because the data are complex and present many unusual features, our primary aim here is to record the data and to correlate the ORD and CD measurements. We also offer some preliminary and tentative interpretations.

MATERIALS AND METHODS

We prepared human carbonic anhydrases B and C from human red cells supplied through the courtesy of Dr. R. B. Pennell of the Blood Characterization and Preservation Laboratory, Jamaica Plain, Massachusetts. The plasma had previously been removed before the red cells were supplied to us. We prepared carbonic anhydrases B and C by fractionation on DEAE-Sephadex as previously described (1, 10). We studied the native proteins in 0.025 M sodium phosphate buffers at pH 7. We prepared and purified urea and guanidine hydrochloride as described by Myers and Edsall (1). The other chemicals that we used were of reagent quality and were not further purified.

We measured the optical rotatory dispersion on a Cary model 00 recording spectropolarimeter in the laboratories of the Harvard Chemistry Department, and circular dichroism on the Jouan dichrograph which had been modified for a 10-fold greater sensitivity (11, 12).

The rotations measured on the Cary instrument were converted to values of the reduced mean residue rotation, [m']

\[ [m'] = \left( \frac{3}{n^2 + 2} \right) \frac{W_m}{100} [\alpha] \]

(1)

Here [α] is the specific rotation at a given wave length, \( W_m \) is the mean residue molecular weight (here taken as 114 for both enzymes), and \( n \) is the refractive index of the solvent at the same wave length. We have taken the values of \( n \), for the most part, from the compilation by Fasman (13) and from other sources given by Myers and Edsall (1). We have assumed the refractive indices of dilute aqueous buffers to be the same as the value for water at the same wave length.

The Jouan dichrograph records directly the difference in absorbance at any wave length for left and right circularly polarized light. This difference in absorbance is then converted to a difference in molar extinction coefficient (\( \epsilon_{L} - \epsilon_{R} \)) on a basis equivalent to [m']; that is, concentrations are expressed as moles of amino acid residues per liter. The mean residue molecular ellipticity obtained is given (11) by

\[ [\theta] = 2.303 \left( \frac{5000}{\nu} \right) (\epsilon_{L} - \epsilon_{R}) \]

(2)

The units of both [m'] and [θ] are degrees cm² per decimole.

RESULTS

ORD Measurements on Native Proteins at pH 7.0 in 0.025 M Phosphate Buffer—The curves in Fig. 1 labeled (exp) show typical ORD patterns of native carbonic anhydrases B and C. (The calculated curves, marked (calc) are discussed below.) The patterns of the data in the 290 to 310 nm range are close to those earlier reported by Myers and Edsall (1) and Rosenberg (3) on both enzymes and by Coleman (2) for Enzyme B. The absolute values of [m'], however, are somewhat lower than those in the other papers cited. Myers and Edsall reported [m'] at 300 nm as -400 for Enzyme B and -493 for Enzyme C in 0.1 M sodium phosphate at pH 7. The values in Fig. 1 are -342 and -380, respectively, in 0.025 M sodium phosphate, also at pH 7. The form of the dispersion curves, however, is almost identical with those reported previously, and the displacement in [m'] between the successive troughs and peaks is numerically nearly the same here as in the other reported studies.

The experimental data at shorter wave lengths are shown in the lower curves on the left of Fig. 1. The principal trough for Enzyme B is at 222 nm, \( [m'] = -2900 \); for Enzyme C it is at 226 nm, \( [m'] = -2900 \). These values are close to those reported by Myers and Edsall (1), who, however, did not extend their measurements below about 220 nm. The present data for Enzyme B show a shoulder at 217 nm, a peak at 206 nm (\( [m'] = -300 \)) and then an abrupt downward turn to \( [m'] = -3000 \) at 200 nm. Below about 196 nm, the steeply rising absorption has hitherto prevented further study. Enzyme C also shows a shoulder at 216 nm, a positive peak at 204 nm (\( [m'] = +2750 \)), and a steep descent to \( [m'] = -1250 \) at 196 nm. Table 1 summarizes the data for the positions and magnitudes of the peaks and troughs in the spectra of both enzymes under various conditions.

Circular Dichroic Spectra of Enzymes B and C in Native State, pH 7—Figs. 2 and 3 show the circular dichroic spectra of En-
Peaks, troughs, and shoulders in short wave length ORD spectra of carbonic anhydrases

The solvent at pH 1.8 was composed of 0.031 M NaH₂PO₄ and 0.069 M H₃PO₄. The solvent at pH 4.0 was 0.025 M NaCl, with sufficient hydrochloric acid to adjust pH to 4.0. The readings recorded here were taken after the protein had been at pH 4.0 for 23 hours. For values observed at earlier times at this pH see Fig. 5.

<table>
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<th>Peak</th>
<th>Trough</th>
<th>Shoulder</th>
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<td>-300</td>
</tr>
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<td>+8450</td>
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<td>-2000</td>
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<td>+2750</td>
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<td>+6905</td>
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<tr>
<td></td>
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<td>222</td>
<td>-2800</td>
</tr>
</tbody>
</table>

Enzyme B exhibits a positive maximum at 235 μm and a negative maximum at about 214 μm. Enzyme C shows a negative maximum, deeper than that of Enzyme D, at 217 μm. Neither spectrum displays the band at 222 μm (nH - π-) which is characteristic of the right handed α-helix (14). If the ellipticity of Enzyme B is subtracted from that of Enzyme C, the resulting difference curve shows a negative maximum at 222 μm with [θ] of -1620 deg. cm² per decimole, which would indicate a possible difference in helix content of about 5%. The choice of difference presentation is, however, arbitrary and the difference between the two enzymes could equally be due to a positive side chain ellipticity band present in Enzyme B and absent in Enzyme C, or to some combination of these possibilities.

ORD of Enzymes B and C in Acid Solution—It has previously been shown (Reference 1, Figs. 1 and 2) that the Cotton effects seen in the native proteins between 250 and 300 μm disappear on denaturation in acid, with an accompanying shift of the whole dispersion curve to more negative values. Fig. 4 shows the ORD and the CD spectra of these enzymes in 0.1 M phosphoric acid.
acid, pH 1.6 to 1.8, in the region below 250 mp. The trough in the ORD pattern, in the 220 to 230 mp region, deepens on acid denaturation and shifts to longer wave lengths. The displacement of $\lambda_{\min}$ for Enzyme B is from 222 in the native protein to 230 to 231 mp at pH 1.8, and $[\theta]$ at the minimum drops from $-2300$ to $-3500$. The shoulder at shorter wave lengths becomes more pronounced than in the native protein with its center near 220 mp. The peak at still shorter wave lengths is now at 194 mp ($[\theta] = +8400$), in contrast with the small negative value of $[\theta]$ in the peak at 200 mp for the native protein. The ORD of the acid-denatured protein can be measured down to shorter wave lengths than that of the native protein because of its lower absorption below 200 mp (see Fig. 7 of Rickli et al. (9)).

The ORD curve of Enzyme C at pH 1.8 gives a flat minimum centered at 227 mp ($[\theta] = -3300$). Thus the shift of $\lambda_{\min}$ to longer wave length on acid denaturation is only 4 mp, as compared with a shift of 8 to 9 mp for Enzyme B. There is a plateau between 214 and 219 mp, and then a steep ascent to a maximum at 193 mp ($[\theta] = +6950$).

Circular Dichroism of Enzymes B and C at pH 1.6 to 1.8—In acid, at pH 1.65, the CD spectra of both enzymes show no ellipticity above 250 mp, as we have pointed out above. In the far ultraviolet, the spectra of both enzymes exhibit well marked negative band maxima at 222 mp for Enzyme B and at 225 mp for Enzyme C (Fig. 4). The former value is the wave length maximum of the $\alpha$-helical $\pi - \pi^*$ transition. It was not possible, because of a very unfavorable ratio of $\theta : c$, to scan at lower wave lengths to determine whether the characteristic “forked” spectrum (14) of the $\alpha$-helix is present. The value of $[\theta]$ at 225 mp for Enzyme C is $-3800$ deg. cm$^2$ per decimole, that for Enzyme B at 222 mp is $-5100$ deg. cm$^2$ per decimole.

Time Course of Acid Denaturation at pH 4.0—The conformational change that results in acid denaturation, for both carbonic anhydrases B and C, sets in at pH values close to 4. The change is manifested by an abrupt upturn in the titration curves, arising primarily from the exposure of imidazole side chains of histidine residues that were shielded in the native protein (15, 16). At the same time, there is a marked blue shift in the ultraviolet absorption spectrum, which gives rise to peaks in the difference spectrum at 235, 285, and 291.5 mp (9, 17). The denatured protein shows a marked tendency to aggregate in the neighborhood of pH 4, and this complicates the study of the reaction. However, the process proceeds at a conveniently measurable rate at this pH and we have made numerous studies of the changes in ORD that occur as a function of time.

The right side of Fig. 5 shows, for carbonic anhydrase B, the changes that occur between 250 and 320 mp in 0.025 M formate buffer. The initial changes are most marked in the 260 mp region, where a rapid shift of $[\theta]$ to more negative values occurs. The Cotton effects in the 280 to 290 mp region are somewhat more persistent, although they have largely disappeared at the end of 1 hour and are completely gone after 3 hours. The left side of Fig. 5 shows the changes occurring at shorter wave lengths, some of which have already been described by Myers and Edsall (1). The trough near 222 mp gradually deepens and shifts to longer wave lengths. At the end of 3 hours, $\lambda_{\min}$ has shifted from 222 mp in the native protein to 226.5 mp. After 23 hours, the minimum is at 230 mp and $[\theta] = -3300$. These values are almost the same as those found for this enzyme at pH 1.8.

At shorter wave lengths, the conformational change at pH 4 leads to a steady shift of the peak to shorter wave lengths and a rise to more positive values, until after 23 hours the value of
Maximum is slightly higher. That for the peak at pH 1.8, although the wave length of the 2-mm cells.

During the entire process of acid denaturation, the rotation at 222 μm remains nearly constant at [m'] = -2150, while the trough at longer wave length and the peak at shorter wave length both steadily increase in absolute magnitude and move apart in position. Although the shoulder at 220 μm is readily apparent in the early stages of acid denaturation of Enzyme B, it becomes less marked with time and is no longer apparent after 23 hours. At this time, the positions and intensities of the extrema at pH 4.0 are almost identical with those at pH 1.8, at which pH a marked shoulder occurs. The crossover point after 23 hours at pH 4.0 is at 214 μm, compared with 207 μm at pH 1.8.

Somewhat similar phenomena are observed during the course of denaturation of carbonic anhydrase C at pH 4.0. The general character of the process is similar to that in Enzyme B, but the changes are less pronounced, with a shift in the position of the minimum of only 4 μm. The plateau seen near 216 μm at pH 1.8 (Fig. 3) is not visible at pH 4, even after 16 hours; instead the curve rises steeply upward from the trough to a peak at 107 to 108 μm ([m'] = -6350) after 16 hours. At pH 1.8, the peak is at 194 μm (see Fig. 4) but the value of [m'] at the maximum is almost the same as at pH 4.0. The crossover point for Enzyme C at pH 4.0 is at 212 μm after 16 hours, compared with 204 μm at pH 1.8. Thus it appears that the acid denaturation at pH 4.0 does not result in entirely the same conformational change as that obtained almost instantaneously at pH 1.8.

Changes of ORD on Denaturation in Alkali. Fig. 6 shows the ORD spectra of carbonic anhydrases B and C at pH 13 at wave lengths below 250 μm. At this pH, the Cotton effects above 250 μm are no longer detected in the ORD spectrum; they are replaced by a smooth featureless dispersion curve. The data of Fig. 6 do not extend below 200 μm since at this pH the intense absorption due to the ionized tyrosyl groups has prevented studies at shorter wave lengths. The most striking feature of the two curves in Fig. 6 is the double minimum displayed by both proteins. Carbonic anhydrase B shows a trough at 232.5 μm ([m'] = -3000), a small peak at 219 μm ([m'] = -2000), and a second shallow trough at 212 μm ([m'] = -2350). For Enzyme C, there is a shallow trough at 230.5 μm ([m'] = -3150), a small peak at 222 μm ([m'] = -2800), and a deeper trough at 209 μm ([m'] = -4400). Although the two troughs are in roughly the same positions for both enzymes, their relative depths are strikingly different. The one near 230 μm is distinctly deeper for Enzyme B, whereas the one at shorter wave length is very pronounced for Enzyme C. The position of this trough at 209 μm suggests at least an apparent analogy with the behavior of polypeptides in the random coil form as illustrated, for example, by the work of Blout, Schmier, and Simmons (18). On the other hand, the troughs in the 230 μm region are, at least in position, close to those characteristic of helical peptides. It is tempting to interpret the data of Fig. 6 as indicating that both carbonic anhydrases B and C at pH 13 consist, at least in part, of a mixture of helix and random coil, the former predominating in Enzyme B and the latter in Enzyme C. In view of the complexity of the situation, however, we suggest such an interpretation only with great diffidence. In any case, it seems clear that there is some important conformational difference between the two enzymes after denaturation at high pH. By contrast, the ORD spectra of Enzymes B and C after denaturation in acid show only relatively minor differences (see Figs. 4 and 5).

Fig. 7 shows in more detail the rotatory dispersion spectra of Enzyme B in the native state, at pH 7.1, 10.8, and 13. At pH 10.8, the titration studies of Riddiford (15) and of Riddiford et al. (16) showed that 2 out of 8 tyrosyl residues have ionized instantaneously and reversibly. There are also indications of some time-dependent but apparently still reversible ionizations of other tyrosyl residues. The ORD spectrum at pH 10.8 at different points differs distinctly from that of the native protein at pH 7. The trough at 222 μm has shifted to 230 and has become slightly shallower, and there is already an indication of an incipient trough at 250 μm, although at this pH it appears as a plateau and shoulder on the rising limb of the curve between 225 and 235 μm. The peak located at 206 μm in the native protein has fallen to a substantially more negative value, although its position is essentially unchanged. Only minor changes are observed in the rotatory dispersion of both Enzymes B and C in the region from 250 to 300 μm.

Further studies of the alterations of Enzyme B in alkaline solutions are shown in Fig. 8, which shows the progress of the observed changes at pH 12.48 and 25°. At the end of 5 hours, the trough at 230 μm is fairly well developed, but the shallower...
Fig. 8. Changes with time in the rotatory dispersion spectrum of carbonic anhydrase B at pH 12.48. The cells used were the same as in Fig. 7.

trough at 213 m, which is so pronounced a feature at pH 13, had scarcely begun to appear at this pH.

Laurent et al. (19) and also Riddiford et al. (10) have shown that Enzyme C is much more susceptible than Enzyme B to alkaline denaturation. Fig. 9 shows the course of the changes in the ORD of Enzyme C at pH 12.33, i.e. at a slightly lower pH than was used in the experiment on Enzyme B in Fig. 8. Here it is clear that the characteristic ORD pattern of the alkali-denatured enzyme is appearing rapidly. The two troughs, the shallower one at 230 m and the deeper one at 210 m, which are characteristic of the alkali-denatured enzyme, are already clearly apparent in the ORD spectrum recorded over the period from 1 to 1½ hours after adjustment to high pH. The two troughs deepen progressively, and at the end of 44 hours the characteristic spectrum is essentially identical with that seen at pH 13. The right side of Fig. 9 shows that the Cotton effects between 270 and 300 m also rapidly disappear. They are for the most part gone in the recording made after 17 min, and only a faint trace of them can be seen after 2 hours.

CD Spectra of Enzyme B at High pH—Fig. 10 shows the CD spectra of Enzyme B at various pH values in alkali. At pH 10.7, the principal negative band near 270 m is shallower than at pH 7.0; it is also displaced to a longer wave length with a broad band maximum centered near 280 m. Furthermore, the positive ellipticity between 240 and 255 m is substantially increased, with [0] at 240 m = 550 deg. cm² per decimole compared to a value of 370 deg. cm² per decimole at pH 7.0. Further increase of pH to 11.5 leads to a further red shift in the negative band to 245 m. Also, at pH 11.5, there is a fairly well marked split in the negative band, already detected at the earliest possible time of measurement (2 min) with a maximum near 295, a slight return toward zero, and then another negative maximum near 270 m. This portion of the spectrum is hardly, if at all, changed over the period 2 min to 71 hours. At about 40 min, the spectrum shows a clear positive maximum at 245 m, [0] = 350 deg. cm² per decimole, which does not noticeably change thereafter with time. This band occurs also, although with diminished height, in the spectra of Enzyme B at all higher pH values. Even at pH 13, after several hours, this band persists, as does a broad negative band between 270 and 310 m. In contrast, it is interesting that the ORD curve at pH 13 is smooth and essentially featureless in this wave length interval.

CD Spectra of Enzyme C at High pH—The data are as yet more limited than for Enzyme B. After 30 min at room temperature at pH 11.5, the short wave length CD spectrum shows a strong suggestion of a minimum at 222 m, with [0] in the order of −3000. The noise level, however, is high and readings are very difficult. Below 220 m, the absorption was too intense to permit CD measurements, but the apparent minimum at 222 m suggests the presence of a- helix and may arise from the a →π- helix band. At pH 11.5, the positive band of the native protein, with its peak at 246 m, has disappeared, and the negative band near 270 m is greatly weakened. There appears to be a small peak, [0] in the order of −50, but the noise in this region of strong absorption is of comparable magnitude. Altogether the
changes in the CD spectra of Enzyme C at pH 11.5, in the region above 240 nm, are much more drastic than in Enzyme B, a result quite in accord with the ORD spectra discussed above, and with other criteria indicating the greater instability of Enzyme C at high pH (16, 19).

Some Effects of Guanidine Hydrochloride on CD and ORD—Myers and Edsall (1) have already reported the disappearance of the Cotton effects above 260 nm in both Enzymes D and C after prolonged exposure to guanidine hydrochloride at concentrations of 2 M or above. Here we report briefly a few further observations. For Enzyme B, in 2 M guanidine-HCl, long wave length ellipticity persists, although diminished in intensity and altered in character compared to that of the native protein. In particular, there is a negative band of ellipticity, \( [\theta] = -50 \), at about 275 nm, and a positive band of ellipticity, +110, at 250 nm. In 4 M guanidine-HCl, the long wave length ellipticity is abolished. In the far ultraviolet, a negative band is observed with a possible trough at 219 to 220 nm and maximum ellipticity close to \(-8100\) deg. cm\(^2\) per decimole. This is substantially greater than the value observed in acid (pH 1.84) with Enzyme B (see Fig. 4).

ORD measurements on Enzyme B in 1 M guanidine-HCl, in 0.025 M phosphate buffer, pH 7.0, showed little change in the Cotton effects at longer wave length, even after 24 hours, or in the trough at 223 nm. At 2 M guanidine-HCl, however, the ORD curve at longer wave lengths appeared smooth and featureless, essentially as shown in Fig. 2 of Myers and Edsall (1). There were no obviously discernible Cotton effects to correspond to the CD bands; thus the CD spectrum reveals details that are obscured in the steep and apparently smooth ORD curve. Also, at shorter wave lengths in 2 M guanidine-HCl, the trough is displaced to 220 nm, with \([\theta] = -3000\) deg. cm\(^2\) per decimole, very close to the values observed for the acid-denatured material at pH 1.8.

The ORD spectrum of carbonic anhydrase C undergoes rapid alteration in the Cotton effects above 260 nm, even in 1 M guanidine-HCl. The Cotton effects are much reduced in less than 15 min, and after 2 hours the curve is steep, nearly smooth, and almost featureless. The trough near 224 nm shifts little in position, but becomes deeper falling from \([\theta] = -2750\) after 2 min to \(-3300\) after 2 hours. Thus Enzyme C is far more stable in this medium. In this respect, the ORD measurements are in full accord with the results from difference spectra (see Fig. 2 of Edsall et al. (4)).

**Computation of ORD Spectra from CD Data; Comparison with Observed ORD Spectra** In the interpretation of data such as ours, there are two aspects of computational analysis. One involves the conversion of the CD data to ORD data. The other involves an attempt to resolve the composite CD spectrum into isolated Gaussian bands, with the aim of identifying the chromophores responsible for the bands. Here we are concerned with the former aspect, which is on a more secure basis, because it involves no assumptions about the number or characteristics of the isolated bands which make up the composite CD spectra.

**Calculation of an ORD spectrum by transformation of the CD spectrum involves a general Kramers-Kronig transform** (Moffitt and Moscowitz (20)).

\[
[m']_\lambda = \frac{2}{\pi} \int_0^{\infty} [\theta]_\nu \left( \frac{\lambda'}{\lambda^2 - \nu^2} \right) d\nu
\]

Values of \([\theta]_\nu\) were tabulated at 0.5 nm intervals. The integral was evaluated with an IBM 7094 computer, by summing the terms

\[
[\theta]_\nu \left( \frac{\lambda'}{\lambda^2 - \nu^2} \right) d\nu
\]

excluding the term where \(\lambda = \lambda'\) from the summation. In contrast with the presentation of the data in Figs. 2 and 3, which are smooth curves, we did not smooth the data for the computations, but determined \([\theta]_\nu\) at each wave length directly by measuring the difference between the recorded deflection of the sample and the base-line. Above 240 nm, we tabulated the data of four experiments in this way and averaged the results; below 240 nm, where readings are more uncertain, we similarly averaged the data from eight experiments. The curve marked \((a)\) in Fig. 1 shows the results of the computation for both enzymes, and the experimental ORD data in the figure can be compared directly with the computed values.

In the near ultraviolet, the form of the computed curve for Enzyme C is quite satisfactory. Each extremum in the observed spectrum is matched by a corresponding one in the computed spectrum. The wave lengths correspond within about 1 nm for the small bands above 265 nm and within 2.5 nm for the band at 265 nm in the observed spectrum. The long wave length negative ellipticity band of both enzymes generates a complex negative Cotton effect in the transforms in the region 250 to 300 nm. This can be considered as being a single smooth negative Cotton effect with extrema at about 262 and 286 nm generated by the major ellipticity band at about 270 nm. On this are superimposed weaker, narrow bandwidth Cotton effects corresponding to the fine structure between 284 and 300 nm in the circular dichroism.

The positive ellipticity at 246 nm in Enzyme C generates a positive Cotton effect with a positive extremum at about 260 nm. This nearly coincides with the positive extremum of the 270 nm band, at approximately 262 nm, and a slight fork is observed in this region of the computed dispersion curve, giving rise to a composite positive extremum around 260 nm. For Enzyme B, this composite peak occurs at a shorter wave length, reflecting the shorter wave length maximum at 233 nm and its greater intensity compared to the 246 nm of Enzyme C.

Each of these positive ellipticity bands gives rise to a negative extremum in the transforms on the short wave length side of their maxima. These cannot be discerned in either the transforms or the observed dispersion curve, although the transform for Enzyme B shows a slight discontinuity at 236 nm. The extrema in the transforms, at 220 to 222 nm for Enzyme R and 232 nm for Enzyme C, are composite negative extrema, arising from the positive ellipticity bands and the intense negative bands around 215 nm. The latter are the major contributors to these extrema.

Difference values between computed and observed curves for both enzymes indicate that the background is due to Cotton effects at shorter wave lengths. For Enzyme C, the difference curve shows no extremum down to 209 nm and the large negative Cotton effect does not, therefore, occur until well below this Lorentz fields at \(\lambda\) and \(\lambda'\), and which is close enough to unity to be omitted.
Table II

Resolution of circular dichroism bands for native carbonic anhydrases at pH 7 in 0.085 M phosphate buffer.

<table>
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<th>( \lambda )</th>
<th>[( \beta )]</th>
<th>( \Delta \beta )</th>
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</tbody>
</table>

Disruption of the native structure by acid, alkali, urea, or guanidine hydrochloride leads to disappearance of these Cotton effects as the protein unfolds. Rosenberg (3) has discussed in detail the magnitude of the effects observed in the proteins. It is clear that the size of the Cotton effects, if expressed in terms of the molar concentrations of tyrosyl and tryptophanyl residues, in proteins such as the carbonic anhydrases, is substantially greater than the relatively small effects observed in the simple aromatic amino acids and their derivatives (27).

We have as yet no knowledge of the detailed three-dimensional structure of the carbonic anhydrases which would permit us to specify the nature of the interactions that give rise to the observed effects. It is natural to inquire, however, concerning the relative contributions of tyrosyl and tryptophanyl residues to the observed patterns. There is some evidence that the negative band centered near 273 \( \mu \)m and the positive band centered at 242 \( \mu \)m in Enzyme C and 235 \( \mu \)m in Enzyme B both arise largely, perhaps entirely, from interactions of tyrosyl residues. The effect of increasing \( \text{pH} \) in shifting the 272 \( \mu \)m band to longer wavelengths (see Fig. 10) in conjunction with the reversible ionization of some of the tyrosyl residues is strongly suggestive of change in the asymmetrical interactions of these tyrosyl residues. Even at \( \text{pH} \) 9.9, we see evidence of change by comparison with \( \text{pH} \) 7. Little change in the ORD is observed in the region from 260 to 300 \( \mu \)m, although the smooth dispersion curve above this region is slightly changed at \( \text{pH} \) 9.9 and \( \text{pH} \) 10.8 from that at \( \text{pH} \) 7. The circular dichroism of Enzyme B at \( \text{pH} \) 10.8 in the region 260 to 300 \( \mu \)m (Fig. 10) is shifted to longer wave lengths and has decreased in intensity. Beychok and Fasman (11) observed such a change for \( L \)-tyrosine and poly-L-tyrosine on dissociation to the phenolate ion. Thus it appears that a part of the 270 \( \mu \)m ellipticity is associated with normally ionizing tyrosine residues which are presumably fully exposed at the enzyme surface.

Helical poly-L-tyrosine (11) at \( \text{pH} \) 11.2 shows three CD bands above 210 \( \mu \)m: negative at 224 \( \mu \)m, with \( R_e = 6.54 \times 10^{-10} \); positive at 248 \( \mu \)m, with \( R_e = 2.66 \times 10^{-10} \); and negative at 270 \( \mu \)m, with \( R_e = 0.91 \times 10^{-10} \). In the random form of this polymer at high \( \text{pH} \), there are only two bands, both positive, centered at 225 and 245 \( \mu \)m; there is nothing corresponding to the positive bands seen in the free amino acid at 274 \( \mu \)m (ionized —OH group) and 294 \( \mu \)m (ionized —OH group). Also, \( L \)-tyrosine has a positive band at 225 to 226 \( \mu \)m, \( [\theta]_2 = 8000 \), when the —OH group is un-ionized, and a negative band at 280 \( \mu \)m, \( [\theta]_2 = -2300 \), when the —OH group is ionized (28).

The bands near 270 \( \mu \)m in Enzymes B and C appear clearly analogous to those of tyrosine. They are opposite in sign to the positive bands observed for both \( L \)-tyrosine and poly-L-tyrosine (11), but of the same sign as that seen in \( N \)-acetyl-L-tyrosine amide (28). The positive band at 249 \( \mu \)m in Enzyme C possibly corresponds to the 248 \( \mu \)m band in poly-\( L \)-tyrosine. Since Enzyme C contains 9 tyrosine residues, of the total of about 266 (10), we must multiply the observed \( [\theta]_2 \) value for the enzyme by a factor of approximately 30, to compare it with the corresponding value of poly-\( L \)-tyrosine. This gives a value of \( +7,500 \text{ deg cm}^2 \text{ per decimole} \) for Enzyme C at 249 \( \mu \)m as against 6,700 for the polymer at 245 \( \mu \)m. The two values are remarkably close, but this of course may be purely a coincidence.
232 nm and is much stronger than in Enzyme C, with $\theta = +576$. Enzyme B contains 8 tyrosine residues out of a total of about 260 (10). Hence the $\theta$ value per tyrosyl residue is approximately +18,700. Interpretation of the bands in this region, however, is greatly complicated by the problem of overlap between the optically active transitions arising from the peptide linkages with those from the aromatic side chains.

The contribution of tryptophan residues to the spectra is even more uncertain. Tryptophan itself has a strong positive CD band at 223 nm, and a weaker positive band, with $\theta = 1155$, at 265 nm (29). Although there is no CD band corresponding to the strong absorption in the 280 to 300 nm region in the free amino acid, it seems highly likely that asymmetrical interactions in the native carbonic anhydrase can give rise to optically active fine structure contributions in the 260 to 300 nm region, a possibility discussed further below.

The ORD and CD patterns at shorter wavelength, from 195 to 240 nm, are notable particularly because of their marked contrast with the patterns characteristic of helical peptides and proteins. Whereas the rotatory dispersion of helical polypeptides shows a trough at 233 nm, with $[\alpha]^195 = -15,000$, and a large positive peak at 198 nm, with $[\alpha]^195 = 65,000$ to 75,000 (30), carbonic anhydrase B gives a low minimum at 222 nm, $[\alpha]^222 = 2,900$, and never attains a positive value of $[\alpha]$ at any wave length above 200 nm. The peak at 206.5 nm is still negative, with $[\alpha]^205 = -250$. In carbonic anhydrase C, the trough is found at 225 nm, $[\alpha]^225 = -2,880$, and the peak at 204 nm is slightly positive, $[\alpha]^204 = +2,750$. These patterns are clearly unlike those of any helical protein, and they do not show any close resemblance to the ORD patterns of peptides in the form of random coils, which show a trough near 205 nm and a peak at approximately 190 nm (30, 31).

Recent studies (5-8) have defined the characteristics of the ORD and CD spectra of polypeptides in the $\alpha$-helical conformation. There is a minimum near 230 nm with a moderately negative value of $[\alpha]$ of the order of $-4,000$ to $-5,000$, and a peak near 208 nm with a value of $[\alpha]$ near 25,000 to 30,000. The corresponding CD bands are shown by the work of Townend et al. (7) to be at 217 nm (negative) and 194 nm (positive). Although we cannot rule out the possible presence of $\beta$ structure in the carbonic anhydrases from these data, our observed spectra certainly do not indicate any obvious resemblance to the $\beta$ polypeptides.

Any attempt to interpret the data for the carbonic anhydrases is necessarily complicated. Rosenberg (27) has shown the presence of strong Cotton effects, centered near 220 nm, in the aromatic amino acids and related compounds; and Beychok (21) has observed corresponding strong CD bands in compounds of this class. It is quite probable that other CD bands at still shorter wavelengths are associated with interactions of the aromatic side chains. Since the contribution of the aromatic residues at longer wavelengths is so conspicuous in the carbonic anhydrases, it is highly probable that the transitions of the aromatic groups at shorter wavelengths make a very large contribution indeed. If these contributions are superimposed upon the patterns that can arise from helix, from random coil, or from $\beta$ structures, the total resulting spectrum will be complex and difficult to disentangle into its component bands. The significance of the bands, even after their positions and rotatory strengths are determined (see Table II), may still be very difficult to infer.

Previous studies of ORD above 300 nm (1, 4, 10) have shown that the Moffitt constant $b_0$ very close to zero if $\lambda_0$ in the Moffitt-Yang equation is taken as 212 nm. Earlier, we (1) have interpreted these data as indicating that the helix content of the native carbonic anhydrase was zero or very small. However, since it is now clear that the Cotton effects of the aromatic side chains must make major contributions to the rotatory dispersion patterns at longer wavelengths, attempts to draw conclusions regarding helix content from the conventional analysis of the Moffitt-Yang equation are open to very serious doubt, as we have already pointed out (4, 10). For similar reasons, we do not propose here to calculate helix content of the native proteins from the values of $[\alpha]$ at 198 or 233 nm.

It is of some interest, however, to consider the differences between the spectra of Enzyme C and Enzyme B, both for ORD and CD. Plots of a difference ORD spectrum, from the data of Fig. 1, show a minimum at approximately 230 nm, which might be due to the presence of a somewhat greater helix content in Enzyme C than in Enzyme B. The depth of the minimum in the difference spectrum would suggest that the former might contain perhaps 5 or 10% more helix than the latter. A similar conclusion is reached from considering the height of the peak in the difference spectrum near 200 nm. The CD spectra are also compatible with such an interpretation. Other interpretations of the difference spectra are possible, however, and we point out these relations only as suggestions.

Acid Denaturation—In the acid-denatured proteins, in which the Cotton effects above 240 nm disappear, there appears to be a greater proportion of the presence of a substantial amount of $\alpha$-helix, perhaps of the order of 20%. At pH 1.6 to 1.8, the trough near 222 nm in Enzyme B shifts to 230 nm and becomes considerably deeper. Correspondingly, the peak near 200 nm shifts to shorter wavelengths and rises from a slightly negative to a moderately positive value. The changes in Enzyme C are less marked but in the same direction. Rickli et al. (9) and Armstrong et al. (10) noted that the value of $b_0$ in the acid-denatured protein is much more negative than in the native protein, being of the order of $-70$ deg. cm$^2$ per decimole. Since the contributions at longer wavelengths from the aromatic side chains disappear on acid denaturation, it seems reasonable to suppose that similar contributions at shorter wavelengths disappear also. The ORD spectrum of the acid-denatured proteins thus assumes a much simpler character than that of the native proteins, and the contributions due to the presence of helix are displayed more clearly. Both the change in the $b_0$ values (10) and the magnitude of the peaks and troughs in the 230 nm and the 185 nm region suggest a helix content of the order of 20%.

The changes in Enzyme C, on acid denaturation, are much less pronounced than those in Enzyme B in the region below 240 nm, although the Cotton effects at longer wavelengths disappear completely. The shift of the trough near 224 nm to longer wavelength lengths is only about 4 nm, compared to a shift of 9 nm in Enzyme B. We note that the $b_0$ value of Enzyme C also changes much less than that of Enzyme B in acid denaturation (10).

The question naturally arises: does acid denaturation result in the formation of helix, or is the helix already present in the native protein, but with its contribution to the rotatory dipe-
cation masked by other bands? Fundamental as this question is, we can at present only ask but not answer it.

Alkaline Denaturation—There are striking differences between the ORD and CD patterns observed in alkaline and those seen in acid. The work of Riddiford (15) has clearly established the fact that Enzyme B is remarkably stable in alkaline solution up to about pH 12 or slightly above. The data of Fig. 8 show that the characteristic ORD spectrum of the native protein is not drastically altered even after 1 hour at pH 12.48. At the end of 5 hours, the trough which was originally at 222 m $\mu$ has shifted to 230 m $\mu$ and a secondary trough near 212 m $\mu$ has just begun to appear. Enzyme C, on the other hand, is definitely more unstable at high pH (16, 19). The data of Fig. 9 show that the characteristic ORD pattern of the alkali-denatured protein, with a shallow trough near 230 and a much deeper trough near 210 m $\mu$, has already developed at pH 12.33 after about 1 hour. Indeed, the spectra of Enzymes B and C at pH 13, as shown in Fig. 6, differ more markedly than the spectra of the two enzymes under any other conditions in which we have compared them. Both spectra show two troughs in approximately the same places, but the one near 230 m $\mu$ is considerably deeper in the spectrum of Enzyme B, whereas the one near 210 m $\mu$ is far deeper in that of Enzyme C. It is tempting to interpret the former trough as being due to the presence of helix and the latter as arising from the existence of some of the protein in a conformation like that of a random coil. If this is indeed the case, then we could say that the random coil form predominated in alkali-denatured Enzyme C and the helical contribution in Enzyme B. In any case, however, we must note that the minimum at 230 m $\mu$ is shallow, with [m'] of the order of -5000, a value which would not in any case suggest the presence of more than 20 to 25% of helix. Unfortunately, the very strong absorption due to the ionized tyrosyl residues at high pH has made it impossible to follow the ORD curves in these alkaline solutions below 200 m $\mu$. We therefore can not estimate the magnitude or position of the positive ORD peak that presumably is present at shorter wave lengths.

When solutions of Enzyme B at pH 13 are neutralized, a striking change is observed in the dispersion curve. Because the alkali-denatured protein becomes insoluble below about pH 9, we have only partially neutralized these solutions. After returning to pH 10.8, we find that the marked trough at 214 m $\mu$ is no longer present. There is an extremum at 231 m $\mu$, [m'] = -3300, and a shoulder at about 220 m $\mu$. In fact, the dispersion is very similar to that for the acid-denatured protein. These findings might cast doubt on the explanation of the 214 m $\mu$ trough as arising from disordered polypeptide. However, it may simply mean that, when the negative net charge on the denatured protein is reduced by lowering the pH, the molecule then assumes a conformation resembling that of the acid-denatured protein.

Possible Origins of Fine Structure at Longer Wave Lengths—The pattern of the CD spectrum between 260 and 310 m $\mu$ suggests a greater complexity than is indicated by the proposed resolution into bands, as outlined in Table II under "Results." Although the background noise in the recordings makes the detection of fine structure in the bands extremely difficult, there is strong indication of at least one shoulder in the CD spectrum of both enzymes near 285 m $\mu$, and possibly a second shoulder near 280 m $\mu$ in Enzyme C. The actual pattern may involve the superposition of a larger number of component bands. In particular, the form of the CD spectrum above 280 m $\mu$ suggests the possibility of a Davydov splitting (32), arising from the interaction of a pair of tryptophan residues, presumably with the indole rings stacked in adjacent parallel planes. This would displace one of the transitions to longer, the other to shorter, wave lengths. With only a small separation of the split transitions, the main transition envelopes would cancel, leaving only the pronounced vibronic component of the indole $Q$ band to be expressed either in the rotary dispersion or in circular dichroism spectra.

Persistent CD Bands in Denatured Enzyme B—The CD spectra of Enzyme B in 2 m guanidine-HCl, as described briefly in the section on "Results," reveal the presence of at least two bands above 240 m $\mu$, which persist in spite of the extensive disorganization of the native enzyme structure that is indicated by ORD and ultraviolet difference spectra (1, 10). Likewise, the data of Fig. 10 for Enzyme B at pH 13 show well marked negative CD between 260 and 315 m $\mu$ and positive CD at shorter wave lengths centered at about 240 m $\mu$. In contrast, the ORD spectra above 250 m $\mu$, in either of these media, give smooth curves above 250 m $\mu$, in which no clear sign of a Cotton effect is apparent to ordinary inspection. These facts emphasize the sensitivity of CD measurements, which clearly can reveal effects that would be overlooked if ORD spectra alone were available.

The studies of Rosenberg (3) are closely related to our own. Insofar as the conditions of our measurements overlap with his, we are in very satisfactory agreement. In his discussion, he has stressed the contribution of the tryptophanyl residues to the Cotton effects associated with the aromatic side chains, whereas we have tentatively interpreted our data with more emphasis on the tyrosyl residues. This difference in emphasis represents no serious disagreement between Rosenberg and ourselves, but it does serve to emphasize the complexity of the situation and the need for further studies, on model systems and on proteins, to clarify the origins of the observed Cotton effects.

Note Added in Proof, September 23, 1966—Since this paper was accepted, one of us (C. L.) has made a series of further CD measurements on both Enzymes B and C, in neutral and acid solutions, with a Durrum-Jasco recording spectropolarimeter (Durrum Instrument Corporation, Palo Alto, California). Resulting CD data for the native proteins show very satisfactory agreement with those obtained on the Jouan dichrograph, as reported in this paper. The data in acid solutions, below about 220 m $\mu$, appear to descend to more negative values as the wave length decreases, rather than turning upward as indicated in Fig. 4. However, the ratio of signal to noise for both instruments in this region of wave length is not greater than 2 to 1, and is often less. Further work in this region of wave length is clearly required, but is very difficult because of the intense absorption of the carbonic anhydrase solutions. We would emphasize here the generally good agreement of the results on the two instruments above 220 m $\mu$.

Acknowledgments—J. McD. Armstrong and J. T. Edsall wish to thank Drs. Peter Urnes, Dan W. Urry, and Eugene S. Pysh of the Harvard Chemistry Department for numerous helpful discussions.

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Optical Rotatory Dispersion and Circular Dichroism of Human Carbonic Anhydrases B and C
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