# Magnetic Circular Dichroism Studies. XVII.[1] Magnetic Circular Dichroism Spectra of Proteins. A New Method for the Quantitative Determination of Tryptophan[2]

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**Abstract:** The MCD spectra of some proteins are reported. Poly-L-lysine exhibits a negative band in the absorption region of the peptide chromophore below 225 nm which presumably belongs to a $\pi-\pi^*$ transition. The intensity of this band was found to be insensitive to conformational changes. In basic solution a small positive band appears at about 232 nm which may be of $n-\pi^*$ parentage. Of the naturally occurring amino acids only tyrosine and tryptophan give intense MCD bands. The long-wavelength positive B term of the $^1L_b$ transition of tryptophan appears in a region (290 nm) where there is negligible overlapping from bands belonging to other amino acids. The intensity of this band seems to be independent of the chromophore location within the peptide chain and its conformation and therefore can be used for the quantitative determination of tryptophan. This is demonstrated for 13 known and well-characterized proteins and the results are in excellent agreement with the literature.

In a series of recent publications we have emphasized that magnetic circular dichroism (MCD) provides useful spectroscopic information about compounds of biological interest such as chlorins,[3] corrins,[4] and nucleosides.[5] The great potential of Faraday effect spectroscopy for biologically important substances has stimulated the interest of several investigators. Particular emphasis has been placed on hemoproteins[6] since their MORD and MCD spectra are dominated by the very intense and characteristic bands resulting from the porphyrin group. Iron-containing proteins without heme have received much less attention. Marlborough, *et al.*,[7] published the MORD spectrum of ferredoxin in the 300–600-nm wavelength region and we have reported on the MCD of xanthine oxidase.[8] However, data have not been reported for metal-free proteins in the absorption region of the aromatic amino acids and the peptide chromophore below 300 nm.

The measurement of natural circular dichroism and optical rotatory dispersion in this wavelength region has contributed significantly to our knowledge about the secondary structure of protein molecules.[9–11] Consequently, an investigation of the kind of information which might be provided by MCD spectroscopy was of interest.

## Experimental Section

A Japan Spectroscopic Co. spectropolarimeter (Durrum-JASCO Model ORD-UV-5) modified to allow CD measurements and to accept a superconducting magnet built by Lockheed Palo Alto Research Laboratoties (Model OSCM-103)[12] was used for CD and MCD measurements. The magnetic field employed was 49.5 kG. The absorption spectra were taken on a Cary 14 spectrophotometer. For pH measurements a Metrohm pH meter E 300 equipped with an EA 120 × electrode was used. D(+)-Camphorsulfonic acid (Eastman Organic Chemicals) was used as the standard compound for calibration of our CD instrument. The compound as obtained by the supplier was found to be only 92% pure. After two recrystallizations from benzene and drying over $P_2O_5$ at 60°, the rotation was $[\alpha]^{25}D$ 21.6° (*c* 1, water) on a Perkin-Elmer 141 polarimeter (lit.[13] 22.1°).

There still exists considerable disagreement in the literature about the $\Delta\epsilon$ value of this substance. Using the Kronig–Kramer transformation, Cassim and Yang[14] calculated the CD curve from ORD data measured on an instrument which had been calibrated against sucrose. They obtained a $\Delta\epsilon_{290}$ value of 2.20, the same value as had been reported by Urry and Pettegrew[15] who carried out absolute circular dichroism measurements using a Cary 1401 attachment. Using a different approach for the Kronig–Kramer transformation, DeTar[13] obtained the value $\Delta\epsilon_{290} = 2.49$. For calibration of our instrument we have chosen a value of 2.20, a setting which is also in agreement with the value $\Delta\epsilon = 3.31$ for isoandrosterone which is the standard recommended by another instrument manufacturer (Jouan). As a secondary standard for the higher wavelength region DeTar[13] has recommended (+)-tris-(ethylenediamine)cobalt(III) iodide hydrate. This compound was synthesized according to the procedure described in the literature.[16] The observed rotation after three crystallizations from water was $[\alpha]^{25}D$ 87.0° (*c* 0.3, water) (lit. 91.9,[13] 89.5,[16] 85.6°[17]). This

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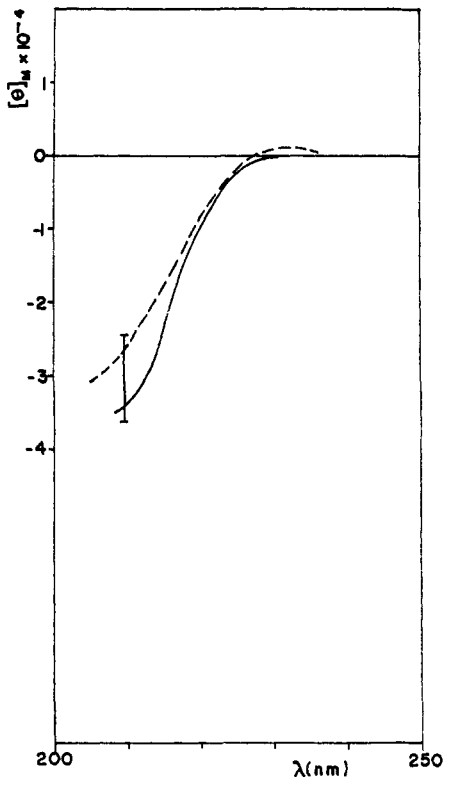


Figure 1. MCD spectrum of poly-L-lysine in 0.1 *N* HCl (—) and 0.1 *N* NaOH (---). $[\theta]_M$ is given for the mean mol wt per residue.

value remained unchanged after further recrystallizations and corresponded to a $\Delta\epsilon_{488}$ value of 1.76 (lit. 1.99,[13] 1.71[17]).

A crucial factor that influences the resolution of sharp CD and MCD bands is the setting of the slit program and the scanning speed. The optimum condition was found to be the setting for which the slit width increased from 0.35 to 0.80 mm between 300 and 250 nm. A scanning speed of 3.3 nm/min was found to be ideal. All measurements were carried out under identical conditions using a cell of 1-cm path length. MCD curves and data have been corrected for natural CD as determined with the same solution but in the absence of the magnetic field. In each figure signal-to-noise ratios are indicated by vertical base.

The reference substances and proteins were obtained from the following sources: L-tryptophan (Nutritional Biochemical Corp.), *N*-acetyl-L-tryptophan methyl ester (Cyclo Chemicals), L-leucyl-L-tyrosinamide hydrochloride monohydrate (Cyclo Chemicals), L-leucyl-L-tyrptophanamide hydrochloride (Cyclo Chemicals), and glycyl-DL-tryptophan (Fluka) were found to be pure by analysis and tlc and were used without further purification.

The following specimens were used without confirming the reported purity: chymotrypsinogen A (bovine) crystallized six times (Miles Laboratories, Inc.), lysozyme (egg white) crystallized six times (Miles Laboratories Inc.), pepsin (swine stomach) crystallized twice from EtOH (Worthington Biochemical Corp.), albumin (bovine) (Mann Research Laboratories), trypsin (bovine pancreas) crystallized twice (Worthington Biochemical Corp.), ovalbumin, crystallized twice (Mann Research Laboratories), casein (bovine milk) (Miles Laboratories Inc.).

Albumin (human serum) (Mann Research Laboratories) was purified over Sephadex G-150. Poly-L-lysine hydrobromide (Serva) (mol wt > 5000) was used after ultracentrifugation with Amicon filters. Glucagon and insulin were a gift from Eli Lilly and Co. Ribonuclease and aspartate transcarbamylase were kindly supplied by Professor G. Hammes (Department of Chemistry, Cornell University). Indole glycerol synthetase (*E. coli*) and tryptophan synthetase A chain (*E. coli*) were obtained from Professor C. Yanofsky (Department of Biology, Stanford University). Guanidine hydrochloride "ultra pure" was purchased from Mann Research Laboratories; 0.01 *M* phosphate buffer (pH 6.90) was used as solvent.

## Results and Discussion

In several review articles the fundamental difference between natural circular dichroism and magnetic circular dichroism has been emphasized.[18-20] Whereas natural CD results from chirality inherent in the molecular structure of compounds of relatively low symmetry, it is the interaction of the external magnetic field with the energy levels of the molecule that leads to the induced optical activity of the Faraday effect. As a consequence, MCD would not be expected to reflect the strong dependency on molecular conformation as is the case with CD.

Poly-L-lysine was chosen as an example of a protein that contains only the peptide chromophore in an accessible wavelength region. Furthermore, this polypeptide is known[21,22] to assume a random coil and an $\alpha$-helix conformation at pH 1 and 10, respectively, as revealed by the intense and characteristic CD Cotton effects at 220–225, 205, and 195 nm. The MCD spectrum (Figure 1) is nearly identical for both conformations and is characterized by a negative curve below 220 nm associated presumably with the $\pi-\pi^*$ transition of the peptide chromophore.[10] This experiment thus demonstrates the insensitivity of MCD to conformational changes insofar as the peptide chromophore is concerned. The lack of a strong MCD band in the $n-\pi^*$ transition region at 220–225 nm is consistent with our previous results[23] that the $n-\pi^*$ transition of carbonyl compounds displays MCD signals of only very low intensity. The weakly positive MCD signal at 232 nm in the basic solution curve may, however, be of $n-\pi^*$ origin. The contributions of the aromatic amino acids histidine, phenylalanine, tyrosine, and tryptophan to the CD spectrum of proteins have in general been considered[24] to be small in the absorption region below 220 nm although exceptions are known.[25] In the wavelength region between 240 and 310 nm the CD Cotton effects of these amino acids have been identified[26] and are clearly visible in several protein spectra.[27] In general, however, the Cotton effects of the aromatic chromophores in this wavelength region are much smaller compared to those of the peptide bond in the 190–225-nm region.

In contrast to their ORD and CD curves, the MCD spectra of these amino acids show some remarkably different features, as illustrated in Table I and Figures 2 and 3. Whereas L-histidine gives no signal above 240 nm, L-phenylalanine and L-cystine exhibit very weak MCD bands at 275 ($[\theta]_M = -0.018 \times 10^3$) and 270

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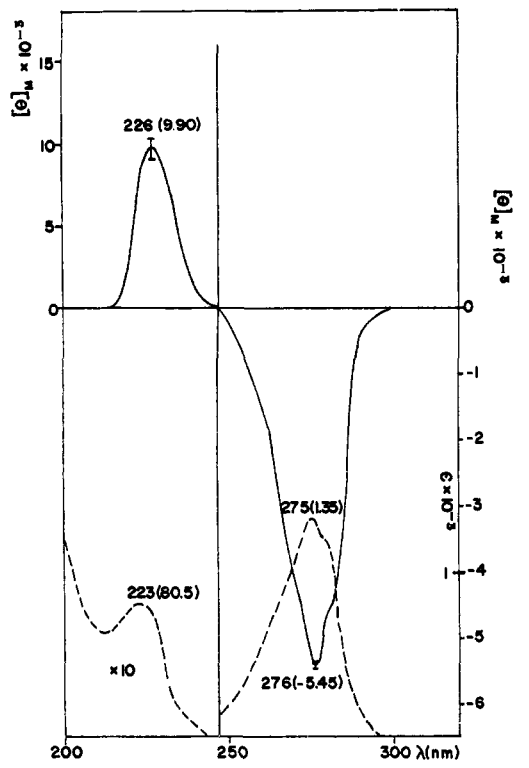


Figure 2. MCD (—) and uv (---) spectra of L-leucyl-L-tyrosinamide hydrochloride monohydrate in 0.01 *M* phosphate buffer (pH 6.9).

nm ($[\theta]_M = -0.35 \times 10^3$), respectively. Tyrosine and, in particular, tryptophan show strong MCD bands that are about 30 and 50 times more intense than the CD Cotton effects[26b,28] and therefore will be discussed

**Table I.** Absorption and Magnetic Circular Dichroism Data for Tryptophan Reference Compounds in 0.01 *M* Phosphate Buffer at pH 6.90

| Compound | Absorption, λ, nm ($10^{-3}\epsilon$) | MCD,[a] λ, nm ($10^{-4}[\theta]_M$) |
|---|---|---|
| L-Tryptophan | 287 (4.76) | 290 ( 4.20 ± 0.06) |
| | 279 (5.71) | 284 ( 0.96 ± 0.02) |
| | | 268 (−3.01 ± 0.06) |
| *N*-Acetyl-L-tryptophan | 287 (4.65) | 290 ( 3.85 ± 0.06)[b] |
| methyl ester | 279 (5.47) | 284 ( 0.79 ± 0.02) |
| | | 267 (−2.90 ± 0.06) |
| L-Leucyl-L-tryptophan- | 287 (4.55) | 291 ( 3.61 ± 0.07) |
| amide acetate | 280 (5.30) | 285 ( 0.65 ± 0.02) |
| | | 268 (−2.76 ± 0.05) |
| Glycyl-DL-tryptophan | 287 (4.58) | 291 ( 3.53 ± 0.05) |
| | 280 (5.32) | 285 ( 0.59 ± 0.01) |
| | | 268 (−2.80 ± 0.06) |
| Lysozyme (hen egg) | 280 (38.30) | 293 ( 3.75 ± 0.06)[c] |

[a] MCD $[\theta]_M$ values (corrected for natural CD) are expressed in deg cm$^2$ dmol$^{-1}$ at a magnetic field of 49.5 kG. [b] A lower value, $[\theta]_M = 3.46 \times 10^4$, has been reported in ref 2 due to a different setting of the slit program. The given values are, however, internally consistent. [c] Contribution per residue of tryptophan based on a mol wt of 14,300. The exact concentration was determined from the absorption at 280 nm, $E_{1\,cm}^{1\%} = 26.5$ given by R. F. Steiner, *Biochim. Biophys. Acta*, **79**, 51 (1964), has been used.

in more detail. The $^1L_b$ absorption band at 275 nm of L-leucyl-L-tyrosinamide (Figure 2) shows a single negative MCD band at the same wavelength position. The partially resolved vibrational fine structure visible as a shoulder at 280 nm in the absorption spectrum is also visible in the MCD spectrum (Figure 2). The $^1L_a$ band at 223 nm gives rise to the positive MCD band at 226 nm. The situation with respect to the indole chromophore is more complicated because the $^1L_a$ and $^1L_b$ bands overlap extensively giving a single absorption band with a maximum at 279 nm (Figure 3). The composite character has been revealed by fluorescence spectroscopy[29] and the assignment of the different vibrational progressions has been carried out recently by Strickland, *et al.*[30] The MCD spectrum shows two oppositely signed B terms which are the result of the mixing of both transitions by the magnetic field.[18-20] The longer wavelength $^1L_b$ transition shows two partially resolved positive MCD bands at 290 and 284 nm associated with the 0–0 and 0 + 730 vibrational components,[30] respectively, whereas the $^1L_a$ band gives a broad negative B term with some vibrational fine structure. It is this important feature of MCD that gives rise to the present analytical application; *tryptophan is the only naturally occurring amino acid that gives a positive MCD band.* Furthermore, this band is almost completely removed from overlapping contributions by other bands. This fortuitous situation suggested the possibility of using MCD as an analytical technique to determine the tryptophan content in proteins. This is an extremely valuable application as will be evident from a brief review of other methods now in use.

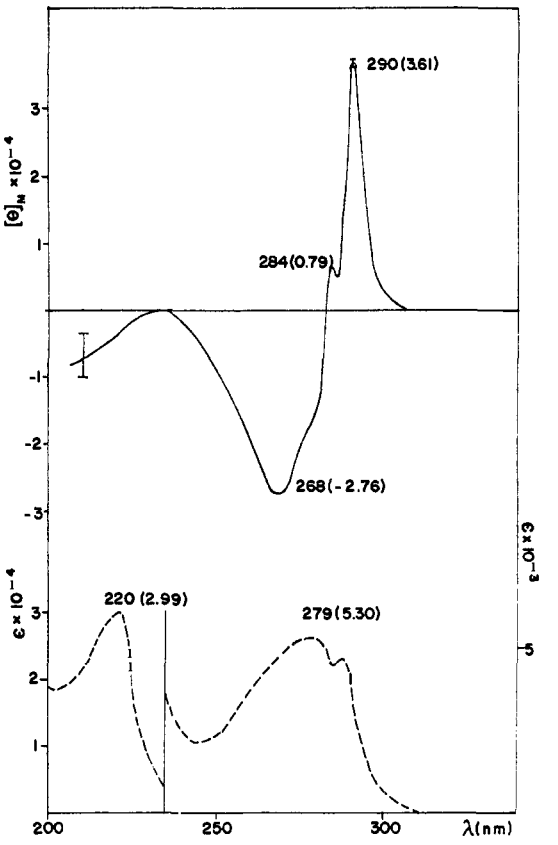


Figure 3. MCD (—) and uv (---) spectra of L-leucyl-L-tryptophanamide acetate in 0.01 *M* phosphate buffer (pH 6.9).

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**Table II.** Tryptophan Content of Proteins Determined by MCD[a]

| Protein | Mol wt | $E_{1\,cm}^{1\%}$ at 280 nm[b] | No. of tryptophan residues/molecule: Lit. | Method used for determination | Determined by MCD | No. of tyrosine residues/molecule |
|---|---|---|---|---|---|---|
| Chymotrypsinogen A (bovine) | 25,670[d] | 20.0[c] | 8[d] | | 8.36 | 4[d] |
| Albumin (bovine) | 64,000[f] | 6.67[e] | 1.85[f] | *s* | 1.93 | 17.9[f] |
| Albumin (human serum) | 69,000[o] | 5.3[o] | 1[p] | *s* | 0.78 | 16[p] |
| Tryptophan synthetase A chain (*E. coli*) | 29,000[q] | 4.5[q] | 0[q] | *t* | 0 | 7.2[q] |
| Indole glycerol synthetase (*E. coli*) | 45,000[h] | 8.2[h] | 2.1[h] | *t* | 2.12 | 14.3[h] |
| Casein (bovine milk) | 26,000 ± 3,000[f] | | 1.32[f] | *s* | 1.38 | 10.6[f] |
| Pepsin (swine stomach) | 36,970[d] | 14.3[i] | 5[d] | | 4.73 | 18[d] |
| Trypsin (bovine pancreas) | 23,560[d] | 13.9[j] | 4[d] | | 3.60 | 10[d] |
| Ovalbumin | 46,000[f] | | 2.71[f] | *s* | 2.65 | 9.4[f] |
| Ribonuclease (bovine) | 13,680[d] | 6.95[k] | 0[d] | | 0 | 6[d] |
| Aspartate transcarbamylase | 310,000[l] | 5.9[l] | 12[q] | *t* | 11.5 | 16.4[q] |
| Glucagon | 3,482[m] | 23.8[n] | 1[m] | | 0.85 | 2[m] |
| Insulin (bovine) | 5,734[r] | | 0[r] | | 0 | 4[r] |
| Poly-L-lysine | >50,000 | | 0 | | 0 | 0 |

[a] All measurements were done in 0.01 *M* phosphate buffer at pH 6.9. The accuracy of the tryptophan values given is believed to be better than 5%. [b] $E_{1\,cm}^{1\%}$ values have been used to calculate the protein concentrations. [c] From D. E. Wilcox, E. Cohen, and W. Tan, *J. Biol. Chem.*, **228**, 999 (1957). [d] From M. O. Dayhoff and R. V. Eck, "Atlas of Protein Sequence and Structure," Vol. 4, The National Biochemical Research Foundation, Silver Spring, Md., 1969. [e] From M. D. Steinman and J. F. Foster, *J. Amer. Chem. Soc.*, **78**, 3652 (1956). [f] From G. R. Tristam and R. H. Smith, *Advan. Protein Chem.*, **18**, 227 (1963). [g] From U. Henning, D. R. Helinski, F. C. Chao, and C. Yanofsky, *J. Biol. Chem.*, **237**, 1523 (1962). [h] From T. E. Creighton and C. Yanofsky, *ibid.*, **241**, 4616 (1966). [i] From H. Edelhoch, *J. Amer. Chem. Soc.*, **79**, 6100 (1957). [j] From E. W. Davie and H. Neurath, *J. Biol. Chem.*, **212**, 515 (1955). [k] From L. M. Sherwood and J. T. Potts, *ibid.*, **240**, 3799 (1965). [l] From J. C. Gerhart and H. Holoubek, *ibid.*, **242**, 2886 (1967). [m] From W. W. Bromer, J. G. Sinn, A. Staub, and O. K. Behrens, *J. Amer. Chem. Soc.*, **78**, 3858 (1956). [n] From W. B. Gratzer, E. Bailey, and G. H. Beaven, *Biochem. Biophys. Res. Commun.*, **28**, 914 (1967). [o] E. J. Cohn, W. L. Hughes, and T. H. Weare, *J. Amer. Chem. Soc.*, **69**, 1753 (1947). [p] H. E. Schultze, N. Heimburger, and G. Frank, *Biochem. Z.*, **336**, 388 (1962). [q] K. Weber, *J. Biol. Chem.*, **243**, 543 (1968). [r] A. P. Ryle, F. Sanger, L. F. Smith, and R. Kitai, *Biochem. J.*, **60**, 541 (1955). [s] Reference 35. [t] Reference 33.

The usual method of amino acid analysis yields very unsatisfactory results since tryptophan is known to decompose under the conditions of acid hydrolysis. Despite some recent improvements by carrying out the hydrolysis in the presence of thioglycolic acid[31] or with $Ba(OH)_2$,[32] spectrophotometric[33,34] or colorimetric[35-38] methods using the intact protein have been most widely applied. Both methods suffer from severe limitations. Absorption contributions from cystine, correction for end absorption, and high tyrosine–tryptophan ratios make the tryptophan determination from the absorption spectrum difficult. The colorimetric method may give equivocal results because of the possibility that the reaction might be incomplete due to differences in reactivity of the tryptophan residues with respect to the location within the macromolecule. In fact, this difference can be used to differentiate between exposed and buried groups.[35] That tryptophan remains the most difficult amino acid to determine underlines the importance of developing a new technique devoid of the above-mentioned complications. In order to determine the scope and limitations of MCD measurements for quantitative tryptophan analysis, several questions had to be answered which will be covered in sequence.

Several of the candidates which we consider as being suitable reference compounds for the tryptophan chromophore are listed in Table I. Since the amino and carboxylic groups are insulated from the indole moiety by two saturated carbons the influence of the particular binding mode of these groups on the MCD spectrum is presumably small. The free amino acid however shows considerable higher $[\theta]_M$ values for all three bands compared to its *N*-acetyl methyl ester and the two dipeptides Leu-Trp and Gly-Trp which have an average value of $[\theta]_M = 3.66 \times 10^4$ for the 290-nm band. The enzyme lysozyme which contains six tryptophan residues and has a molecular weight of 14,300 (see footnote *d* in Table II) has been included in Table I because it is available in a highly purified form. The $[\theta]_M$ value per tryptophan residue is only 3% higher than the one given above. This provides good evidence for the assumption that the contributions from the different indole groups within a peptide chain are almost equal and additive. Consequently a value of $[\theta]_M = 3.70 \pm 0.15 \times 10^4$ has been used for determining the tryptophan content of the proteins listed in Table II. However, there still remains the possibility that the indole chromophore might be perturbed by closely lying groups due to a particular folding of the peptide chain. Furthermore, the difference of polarity in the interior of the protein compared to a location where the chromophore is exposed to the solvent might be expected to influence the intensity of the MCD band. Unfolding of a protein by strong urea or guanidine hydrochloride solutions usually results in a blue shift of the absorption band at around 280 nm and small intensity changes.[39]

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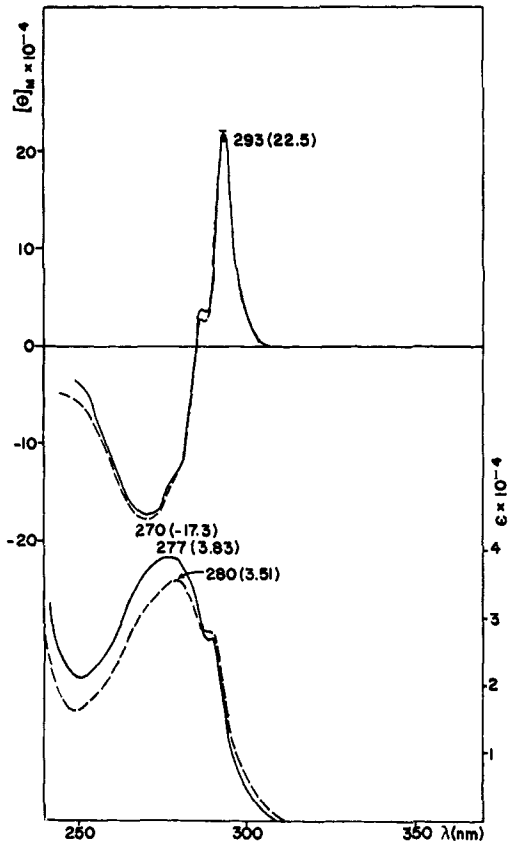


Figure 4. MCD (upper curves) and uv (lower curves) of lysozyme in 0.01 *M* phosphate buffer (pH 6.9) (—) and 5 *N* guanidine hydrochloride solution (---) after standing at room temperature for 36 hr.

From difference absorption spectra it has been concluded[40] that at least one of the six tryptophan residues of lysozyme is buried in the protein and becomes exposed after denaturing in 8 *N* guanidine hydrochloride. In Figure 4 the MCD spectra of both the native and denaturated forms of lysozyme are reported. Virtually no change in the intensity of the 293-nm MCD band is observed, thus providing good evidence that the band intensity seems to be unaffected by the conformation of the protein.

Since the absorption spectra of tryptophan and, in particular, tyrosine are pH dependent,[41] the influence of this parameter on the MCD spectrum was determined. Whereas no change was observed in the MCD spectrum of tryptophan between pH 1 and 12, the tyrosine MCD spectrum is highly sensitive to pH changes as shown in Figure 5. At pH values higher than 8, a shoulder appears on the long-wavelength side of the main band which increases in intensity and leads to the negative band at 292 nm at pH 12. These changes are caused by the ionization of the phenolic chromophore. In order to keep the overlapping contribution of tyrosine to the 290-nm tryptophan band at a minimum, it is obviously necessary to carry out the measurements at a pH below 8.

The error due to overlapping contribution from the negative tyrosine MCD band is comparatively small (approximately 1% when the tyrosine–tryptophan ratio

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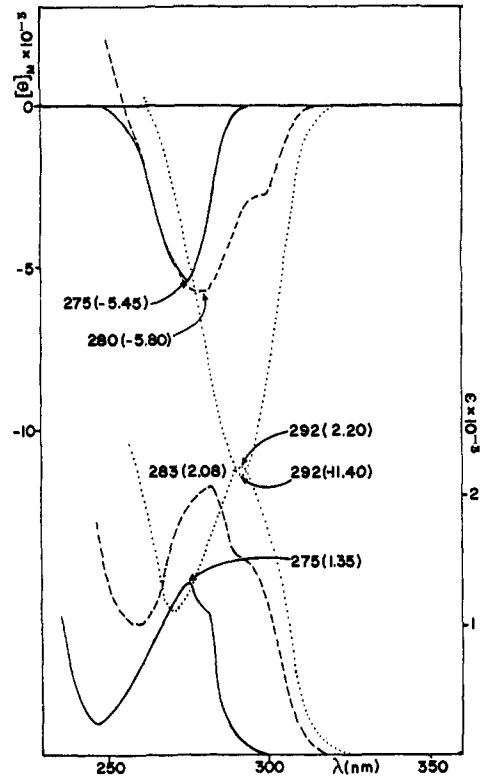


Figure 5. pH dependency of MCD (upper curves) and uv (lower curves) spectra of L-leucyl-L-tyrosine amide hydrochloride monohydrate: pH 1–7 (—), pH 9.5 (---), pH 12.1 (· · ·).

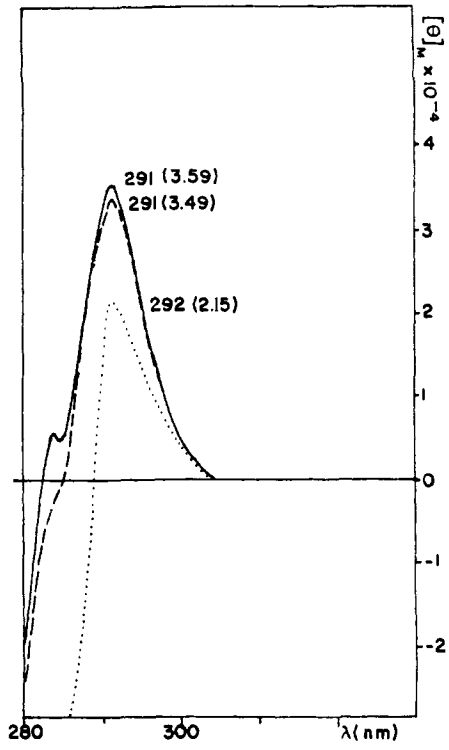


Figure 6. Influence of the concentration of L-leucyl-L-tyrosinamide on the MCD band intensity at 291 nm of L-leucyl-L-tryptophanamide in 0.01 *M* phosphate buffer (pH 6.9). The molar ratios are: 1:1 (—), 4.4:1 (---), 35:1 (· · ·).

is 1:1), but it cannot be neglected in those cases for which the tyrosine–tryptophan ratio is high. The effect of increasing tyrosine concentrations on the intensity of the 290-nm MCD band can be seen in

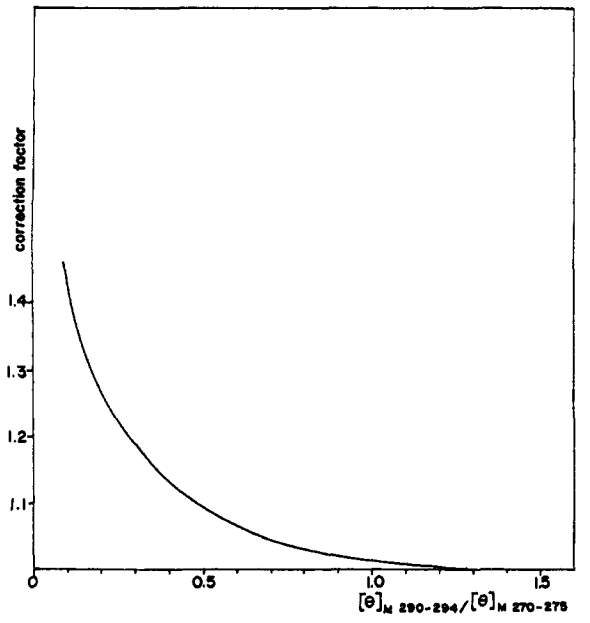


Figure 7. Empirical relationship between the peak ratio of the 290–294- and 270–275-nm MCD bands and correction factor for the intensity of the 290–294 MCD band in proteins.

Figure 6. At a ratio of 4.4:1 the small positive band at 284 nm is only observable as a deflection, whereas the intensity of the 290-nm band remains almost unchanged. Further increase of the tyrosine concentration reduces the intensity of the 290-nm band gradually. At a 35-fold molar excess the intensity has decreased by 40%. In naturally occurring proteins the tyrosine–tryptophan ratio usually does not exceed 15. Since the intensity ratio of the bands at 290 and 275 nm can be assumed to be approximately proportional to the extent of overlapping, it is possible to account for the tyrosine contribution in proteins of unknown composition by reference to the correlation of the empirical correction factor for the intensity of the 290-nm band as shown in Figure 7.

In order to test the applicability of the method we carried out tryptophan determinations on a number of proteins whose molecular weight and amino acid composition are well known. As can be seen from Table II, the resulting values are in close agreement with those given in the literature. Finally, it should be pointed out that the method is quite sensitive and can be carried out with small amounts of material. Depending on the tryptophan content, protein concentrations of 0.1–0.5 mg/ml have been used in our studies. Since the sensitivity of the method is directly proportional to the magnetic field strength, substitution of a cheaper permanent magnet (10 kG) for a superconducting one (50 kG) would decrease the sensitivity accordingly.

In conclusion it can be stated that magnetic circular dichroism in contrast to natural circular dichroism does not provide conformational information in proteins but the technique can be applied very successfully as an analytical tool for the determination of tryptophan in the intact protein. This is due to a sharp positive MCD band in a wavelength region in which there is minimal interference by other potential contributions. Further work is under way in our laboratory to explore these and other analytical applications of MCD.

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