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Tryptophan Quantitation by Magnetic Circular Dichroism in Native and Modified Proteins†

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ABSTRACT: Magnetic circular dichroism (MCD) has been used to determine the tryptophan content of proteins. The intense positive band near 293 nm, uniquely characteristic of tryptophan, allows the direct and quantitative measurement of this amino acid with high sensitivity and accuracy under conditions which preserve the integrity of the native protein structure and function. Tryptophan determinations of 17 proteins have been made and the results compared with those obtained by other recognized procedures. Solvent perturbation and denaturation studies indicate that the environment of the indole chromophore minimally affects the intensity of the band but significantly shifts the wavelength maximum necessitating the identification of the exact wavelength of λ_{max} in the quantitation process. The effects of tryptophan side chain and indole ring substituents have also been examined. The MCD spectra of ring-substituted indoles vary widely and can be used to identify and quantitate tryptophan derivatives and to monitor the chemical modification of this residue in proteins. The reaction of tryptophan with *N*-bromosuccinimide and of tryptophan and the tryptophyl residues in lysozyme with *o*-nitrophenylsulfenyl chloride have been examined in this regard.

Much of the remarkable progress in protein chemistry over the past years has been due to the development of accurate and convenient methods for the qualitative and quantitative analysis of amino acids. Determination of tryptophan, however, has been a persistent problem. This is particularly unfortunate since this residue contributes significantly to the optical, stereooptical, and fluorescent properties of proteins. Tryptophanyl residues have been postulated to be involved in enzyme catalysis and substrate binding (Glickson *et al.*, 1971); Robbins and Holmes, 1972), and their spectral characteristics are considered to be important gauges of protein conformation (Donovan, 1969; Herskovits and Sorenson, 1968a,b; Wetlaufer, 1962; Kauzmann, 1959).

We have previously reported (Holmquist, 1971) on the

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magnetic circular dichroic (MCD)[1] properties of proteins and pointed to the presence of a discrete and prominent magnetically induced dichroic band between 290 and 300 nm characteristic of tryptophan. Further, studies of tryptophan containing peptides and proteins and of indole derivatives have demonstrated that this MCD band can serve as a convenient and nondestructive means to quantitate this residue in native proteins. MCD can also monitor the consequences of certain chemical modifications of tryptophan which in turn can aid in studying the participation of this residue in catalysis and/or binding of substrates, inhibitors, and coenzymes.

Our initial presentation of portions of this work coincided with that of Barth *et al.* (1971) who independently evaluated the use of MCD in the quantitative assessment of tryptophan in proteins with analogous conclusions.

Materials. Crystalline pepsin (porcine), α-chymotrypsin (bovine pancreas), chymotrypsinogen A (bovine pancreas), lysozyme (chicken egg white), DIP-trypsin (bovine pancreas), aldolase (rabbit muscle), carboxypeptidase A (bovine pancreas), purified deoxyribonuclease (bovine pancreas), carbonic anhydrase (bovine erythrocytes), avidin (egg white), ribonuclease A (bovine pancreas), and staphylococcal nuclease were all obtained from Worthington Biochemical Corp. Crystalline *B. subtilis* neutral protease was purchased from Seikagaku Kogyo Co., Japan. Thermolysin (*B. thermoproteolyticus*) (Calbiochem) was recrystallized twice before use (Latt *et al.*, 1969). Twice recrystallized subtilisin (Novo) was obtained from Novo Industries, three times recrystallized α-lactalbumin from Gallard-Schlesinger Chem. Mfg. Corp. and crystalline bovine serum albumin from Pentex Inc. The following materials were used as supplied: *N*-acetyl-L-tyrosine amide, L- and D-tyrosine, *o*-nitrophenylsulfenyl chloride, 5-nitroindole, 2-indolecarboxylic acid, 1-indolecarboxylic acid ethyl ester, L- and D-phenylalanine (Sigma Chemical Corp.); glycyl-L-tryptophan·H_2O, L-tryptophan amide hydrochloride·H_2O, L-phenylalanyl-L-tryptophan amide hydrochloride·H_2O, L-tryptophyl-L-tryptophan, tryptophan diketopiperazine, *N*-acetyl-L-phenylalanine amide, *N*-acetyl-L-tryptophan amide, *N*-acetyl-L-tryptophan ethyl ester (Cyclo Chemical Co.); carbobenzoxy-L-tryptophan, L- and D-tryptophan, *N*-acetylindole (K and K Labs). Indole (Eastman Chemicals) was recrystallized from ethanol–water, *N*-bromosuccinimide (Eastman Chemicals) from water, and *d-10*-camphorsulfonic acid (Eastman Chemicals) from benzene followed by drying under vacuum over P_2O_5. Indoline (Eastman Chemicals) was redistilled before use. Urea and guanidine hydrochloride were ultra pure grades (Mann Res. Labs). All other materials were reagent grade, and deionized distilled water was used throughout.

Methods. MCD measurements were performed using a Cary 61 circular dichroic spectrophotometer equipped with a Varian Model V4145 super-conducting magnet, energized by a Varian V4106 power supply. Magnetic field strengths were varied from 0 to 47 kG using the current-field calibration constant supplied with the magnet (0.6 A/kG). For routine work a field of 40 kG was used. In general, scans were made at a speed of 0.2 nm/sec with a spectral bandwidth of 2 nm. Appropriate examinations of the effects of scan speeds, spectral bandwidths, and instrument pen period time constants were made to ensure optimal spectral resolution. Circular dichroism (CD) measurements were made either with a Cary 61 or with a Cary 60 spectropolarimeter equipped with a Cary 6001 CD attachment. Cylindrical neckless cuvettes constructed of Suprasil of 1- and 2-cm path lengths with volumes of from 1 to 4 ml were used for all studies. Absorbancies of all samples were maintained below 2.0 absorbance units. Absorption spectra were obtained with a Cary 14 spectrophotometer equipped with a Cary tungsten–iodine high-intensity light source. Unless otherwise stated, all measurements were made at ambient temperatures of 21–23°.

In a typical experiment a solution of the sample to be analyzed was placed in a cuvette and its absorption spectrum measured with a Cary 14 spectrophotometer using air as the reference. The cuvette was transferred immediately to the Cary 61 to record the corresponding MCD spectrum and finally to the Cary 60 for the CD spectrum. The same cuvette was then filled with a blank solution containing all components save for the material under study and the base-line spectra were measured by the same three techniques without changing instrumental parameters or settings.

CD data are reported as molar ellipticities, $[\theta]$, obtained from $[\theta] = 100(\theta)/bM$ in units of deg cm²/decimol, where θ is the ellipticity in degrees as obtained directly from instrumental readings, b is the path length, and M the molar concentration of the sample. Values of $[\theta]_M$, the molar magnetically induced ellipticity, were determined from the difference between the ellipticity in the presence of the magnetic field and the natural CD and are normalized to the field of 1 kG with units of deg cm² decimol^{-1} kG^{-1}. We have followed the suggestion of McCaffery *et al.* (1967) defining the Verdet constant of water as negative to serve as the basis of the sign convention for $[\theta]_M$.

The CD intensity was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid, $\theta^{1\ mg/ml}_{289\ nm} = 0.308°$ (Cassim and Yang, 1969).

The MCD intensity was calibrated with a 0.05 M aqueous solution of Johnson–Mathey "specpure" $CoSO_4 \cdot 7H_2O$ which exhibited a rotation of −0.122° at 510 nm (1 cm path length) when measured at 40 kG ($[\theta]^{1\ cm}_{M,\ 510} = -6.1$ deg cm² decimol^{-1} kG^{-1}), in good agreement with the value determined previously for this material (McCaffery *et al.*, 1967). MCD calibrations were performed periodically after current ramp-down into persistent mode operation to ensure that no loss of field occurred during this process. Routine use was also made of the ratio of ellipticity of the positive MCD peak near 290 nm of *N*-acetyl-L-tryptophan amide to its absorption at 280 nm measured on the Cary 14 (0.0621° at 43.3 kG). This ratio served as an additional control of reproducibility both of the ellipticity and of the magnetic field.

Protein concentrations were determined by absorption using the following absorptivities and molecular weights: ribonuclease, $\epsilon_{277.5}$ 9.8 × 10³, mol wt 13,700 (Wetlaufer, 1962); α-chymotrypsin, $A^{1\%}_{282} = 20.4$, mol wt 25,000 (Morimoto and Kegeles, 1967); chymotrypsinogen A, $A^{1\%}_{280} = 21$, mol wt 25,100 (Desnuelle and Rovery, 1961); DIP-trypsin, $A^{1\%}_{280} = 15.4$, mol wt 23,985 (Keil, 1971); pepsin, ϵ_{278} 5.1 × 10⁴, mol wt 33,500 (Fruton, 1971); *B. subtilis* neutral protease, $A^{1\%}_{280} = 13.6$, mol wt 44,700 (McConn *et al.*, 1964); carboxypeptidase A, ϵ_{280} 6.42 × 10⁴, mol wt 37,400 (Simpson *et al.*, 1963); deoxyribonuclease, $A^{1\%}_{280} = 12.3$, mol wt 31,000 (Lindberg, 1967); lysozyme, $A^{1\%}_{280} = 25.5$, mol wt 14,307 (Praissman and Rupley, 1968); avidin, $A^{1\%}_{282} = 15.4$, mol wt 66,000 (Green and Toms, 1970); thermolysin, $A^{1\%}_{280} = 17.65$ (Ohta *et al.*, 1966), mol wt 34,600 (Titani *et al.*, 1972); α-lactalbumin, $A^{1\%}_{280} = 20.1$, mol wt 14,437 (Kronman and Andreotii, 1964); carbonic anhydrase, ϵ_{280} 5.6 × 10⁴, mol wt 29,500 (Lindskog, 1960); aldolase, $A^{1\%}_{280} = 9.38$ (Donovan, 1964), mol wt 158,000 (Kawahara and Tanford, 1966); subtilisin (Novo), ϵ_{278} 3.31 ×

[1] Abbreviations used are: NBS, *N*-bromosuccinimide; NPS, *o*-nitrophenylsulfenyl chloride; MCD, magnetic circular dichroic.

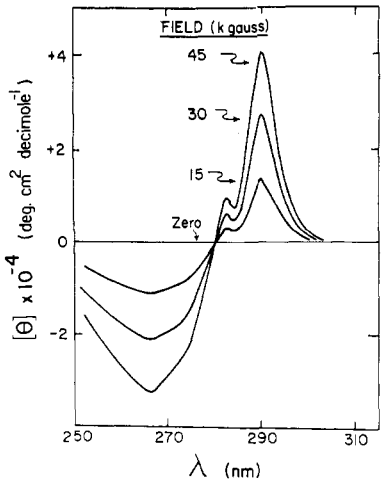

FIGURE 1: MCD spectra of L-tryptophan as a function of the magnetic field. Conditions: pH 7.5, 0.05 M Tris, 25°.

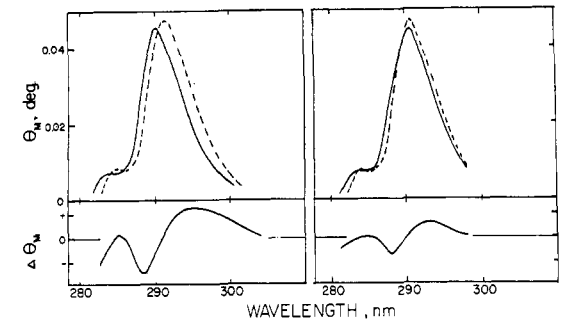

FIGURE 2: (Above) Ellipticity of *N*-acetyl-L-tryptophan amide, 1.36×10^{-4} M–0.05 M Tris (pH 7.5)–0.1 M NaCl, measured at 40 kG. (Upper left) (—) water, (- - -) 6 M guanidine hydrochloride. (Upper right) (—) water; (- - -) 20% ethylene glycol. (Below) Corresponding difference spectra calculated by subtracting the spectrum in water from the spectrum in the respective solvent.

10^3, mol wt 27,500 (Neet *et al.*, 1968); staphylococcal nuclease, $A_{280}^{1\%} = 9.3$, mol wt 16,807 (Fuchs *et al.*, 1967); bovine serum albumin, $A_{280}^{1\%} = 6.60$, mol wt 66,000 (Kolthoff *et al.*, 1965).

Concentrations for low molecular weight compounds were determined by weight with the exceptions of the following which were based on absorption: L-Trp-L-Trp diketopiperazine, ϵ_{280} 11,140; *N*-acetyl-L-tryptophan amide, ϵ_{280} 5520 (Edelhoch *et al.*, 1968); L-tryptophan, ϵ_{282} 5500 (Bailey *et al.*, 1968).

Determinations of pH were made on a Beckman Expandomatic pH meter equipped with a Radiometer combined electrode Model GK 2321 C standardized with Thomas pH 4 and 7 buffers. The pH titration curve of *N*-acetyl-L-tyrosine amide was determined by titrating 50 ml of a 3.7×10^{-4} M solution, in 0.1 M NaCl, preadjusted to pH 4.0 with 6 M HCl using 0.1 M NaOH and a microsyringe buret. At each increment of pH a 1-ml aliquot was withdrawn and its absorption and MCD spectra were measured. The aliquot was then discarded and a new one taken at the next pH.

The tryptophyl residues of lysozyme were chemically modified with *o*-nitrophenylsulfenyl chloride by the method of Scoffone *et al.* (1968).

Results

L-Tryptophan exhibits a characteristic MCD spectrum consisting of a large positive band with a maximum at 289.6 nm, a smaller positive peak at 282.2 nm, and a broad region of negative ellipticity centered near 267 nm with intensities, $[\theta]_M$, of 875, 236, and −617 deg cm² decimol⁻¹ kG⁻¹, respectively (Figure 1). In contrast, the absorption spectrum consists of a broad envelope of absorption with maxima at 288, 279, and 272 nm. The intensities of all magnetically induced bands are linear functions both of concentration and magnetic field strength. Compared to its MCD, the CD of L-tryptophan is negligibly small, $[\theta]_{280} = \sim 50$ deg cm² decimol⁻¹ (Myer and MacDonald, 1967), and would not be discernible when plotted on the scale of Figure 1. The D isomer of tryptophan, tryptophan-containing peptides, and other amino and carboxyl substituted derivatives of tryptophan display spectral characteristics closely similar to those of L-tryoptophan itself, both in regard to wavelength maxima and band intensities (Table I). Thus, just as these side-chain substitution reactions of tryptophan only minimally affect the near-uv absorption of the aromatic chromophore (Donovan *et al.*, 1961) they also only minimally affect the MCD spectra of these derivatives.

TABLE 1: MCD of Tryptophan and Tryptophan Derivatives.[a]

Compound	λ_{max} (±0.2 nm)	$[\theta]_M$[b]
L-Tryptophan	289.6	875
D-Tryptophan	289.6	875
N-Acetyl-L-tryptophan	290.8	798
Glycyl-L-tryptophan	290.8	774
L-Tryptophyl-L-tryptophan	290.5	1560
Carbobenzoxy-L-tryptophan	291.0	772
L-Tryptophan amide	289.6	850
L-Phenylalanyl-L-tryptophan amide	290.5	792
L-Tryptophyl-L-tryptophan diketopiperazine	290.5	1630
N-Acetyl-L-tryptophan amide	290.4	770
N-Acetyl-L-tryptophan ethyl ester	290.4	791

[a] Measured at pH 7.5, 0.05 M Tris–0.1 M NaCl, 23°. [b] Concentrations determined as explained in the Experimental Section.

Various solvents are known to affect the absorption spectrum of tryptophan, and effects similar in magnitude and type are observed in the MCD spectra of tryptophan and its derivatives. In water the maximum of the positive magnetic ellipticity band of *N*-acetyl-L-tryptophan amide is at 290.4 nm (770).[2] In aqueous solutions containing 20% methanol, 20% dimethyl sulfoxide, 8 M urea, 20% ethylene glycol, or 6 M guanidine hydrochloride (see Figure 2 for the latter two) these values change to 290.6 nm (772), 291.5 nm (786), 291.5 nm (798), 290.8 nm (772), and 291.5 nm (816), respectively. Among these solvents 6 M guanidine hydrochloride maximally displaces the peak position from 290.4 to 292.1 nm with an increase in $[\theta]_M$ of approximately 46 deg cm² decimol⁻¹ kG⁻¹, *i.e.*, 6%. The difference MCD of *N*-acetyl-L-tryptophan amide, obtained by subtracting its spectrum in water from those determined in 6 M guanidine hydrochloride and 20% ethylene glycol, are also shown in Figure 2. The MCD spectra of aqueous solutions of *N*-acetyl-L-tryptophan amide and *N*-acetyl-L-tryptophan ethyl ester are the same in 10^{-2} M sodium hydroxide and in 10^{-2} M hydrochloric acid.

[2] Numbers in parentheses adjacent to wavelength maxima of MCD bands are the molar magnetic ellipticities in deg cm² decimol⁻¹ kG⁻¹.

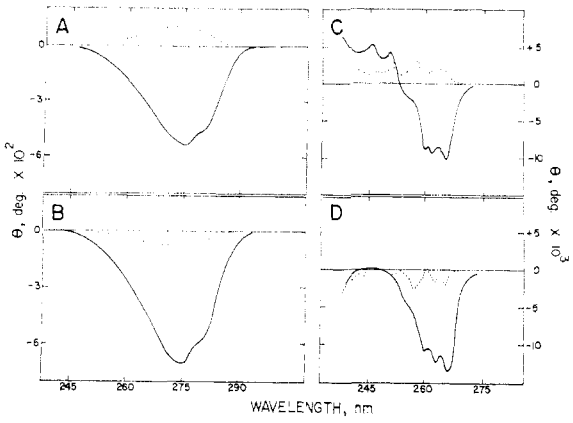

FIGURE 3: Ellipticity of the L and D isomers of tyrosine and phenylalanine in the presence (—) and absence (- - -) of a 40-kG field measured at pH 7.5, 0.1 M NaCl–0.05 M Tris. (A) L-Tyr, 1.16×10^{-3} M; (B) D-Tyr, 1.08×10^{-3} M; (C) L-Phe, 4.5×10^{-3} M; (D) D-Phe, 3.82×10^{-3} M.

Tyrosine and phenylalanine also exhibit distinct MCD spectra between 230 and 300 nm (Figure 3). After correction for the CD, the MCD spectra of the D isomers of both these amino acids are superimposable on those of the respective L isomers. The MCD spectrum of L-tyrosine consists of a negative band at 275 nm (125) and a shoulder near 278 nm (102) which correspond to transitions observed in the absorption spectrum. The maximal ellipticity at 40 kG is approximately five times greater than that in the absence of a magnetic field (Figure 3). The MCD spectrum of tyrosine changes markedly on ionization of the phenolic hydroxyl group. Titration of *N*-acetyl-L-tyrosine amide from pH 4 to 11.5 generates a negative band at 295 nm with twice the magnitude of that at the 275-nm band of the protonated form, with an isosbestic point at 279 nm (Figure 4). An MCD–pH titration (Figure 4, insert), yields a pK_a of 9.9 based on measurements of 295 nm in agreement with the spectrophotometric pH titration curve obtained at the same wavelength.

The MCD spectrum of phenylalanine is multibanded with three sharp, negative transitions of low intensity centered at 267.2 (18), 260.8 (17), and 255 nm (6.3) (Figure 3), closely similar to the spectral detail of its weak absorption spectrum.

Neither cystine, cysteine, histidine, nor free imidazole exhibits significant magnetically induced ellipticities between 230 and 350 nm.

The characteristics of the MCD spectra of tryptophan and its derivatives enable the determination of tryptophyl residues of proteins. Figure 5 shows pertinent segments of the MCD and CD spectra of ribonuclease, staphylococcal nuclease, the neutral protease from *B. subtilis*, and chicken egg-white lysozyme containing 0, 1, 4, and 6 tryptophyl residues, respectively. The magnitude of the positive MCD band, characteristic of tryptophan near 290 nm, is a linear function of tryptophan content. This relationship holds for a series of proteins with tryptophan contents varying from 0 to 8 residues per mole (Figure 6). The slope of the correlation plot obtained by the method of least squares is 777 deg cm² decimol^{-1} kG^{-1} per tryptophyl residue with a correlation coefficient of 0.9993. This slope is virtually identical with the magnetic ellipticity of *N*-acetyl-L-tryptophan amide and of *N*-acetyl-L-tryptophan ethyl ester, 770 and 791 deg cm² decimol^{-1} kG^{-1}, respectively.

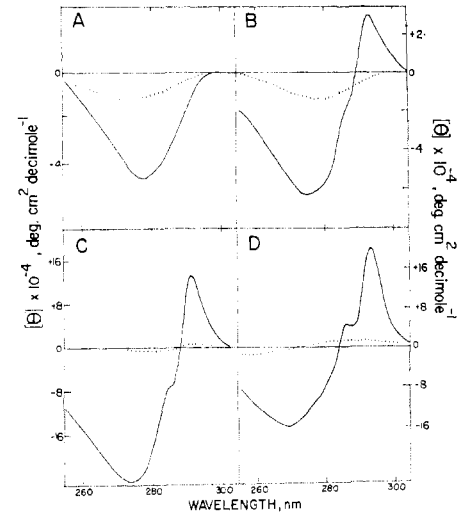

FIGURE 5: Ellipticity of proteins in the absence (- - -) and presence (——) of a 40-kG magnetic field. (A) Ribonuclease, 5.76×10^{-5} M, (B) staphylococcus nuclease, 4.93×10^{-4} M, (C) *B. subtilis* neutral protease, 1.42×10^{-5} M, and (D) lysozyme, 2.74×10^{-5} M. Conditions: pH 7.5, 0.05 M Tris–0.1 M NaCl.

Table II summarizes the results of studies employing a large series of purified proteins. Calculation of tryptophan content is based on an MCD value of 777 deg cm² decimol^{-1} kG^{-1} per tryptophyl residue, corrected for CD and tyrosine content.

The MCD spectra of the proteins were generally measured in 0.05 M Tris–0.1 M NaCl (pH 7.5) though differences were not observed in other buffers, *e.g.*, phosphate and morpholinoethanesulfonic acid, over the pH range from 6 to 8.

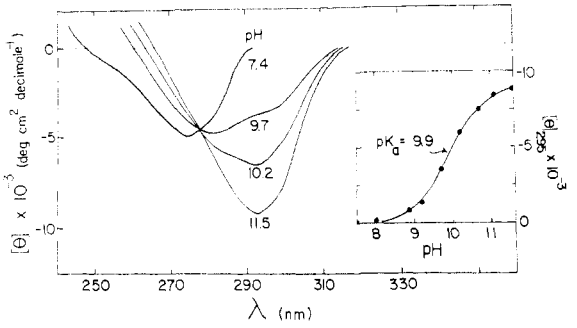

FIGURE 4: Spectral pH titration of *N*-acetyl-L-tyrosine amide, 3.66×10^{-4} M, performed as described in the text at a field of 40 kG at room temperature. Ionic strength was held constant at 0.1 M with NaCl. Inset: corresponding titration curve obtained from the MCD spectra measured at 295 nm.

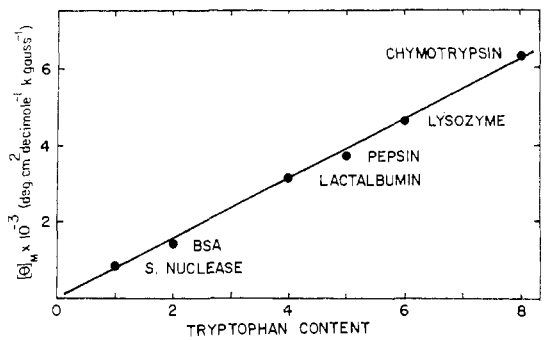

FIGURE 6: Correlation of the tryptophan content of well-characterized proteins with the molar magnetic ellipticity of their positive band at 292–293 nm. The line is the least-squares fit to the points forced through the origin.

TABLE II: Tryptophan Contents of Proteins.

Protein[a]	λ_{max} (±0.2 nm)	No. of Trp Residues: Found by MCD[b]	No. of Trp Residues: Lit.	Ref
Riobnuclease		0	0	Hirs *et al.* (1960)
Lysozyme[c]	292.8	5.96, 5.88, 6.20, 6.02	6	Canfield (1963)
Pepsin	292.6	4.92, 4.90, 4.91, 5.08, 5.07, 4.95	5	Fruton (1971)
Chymotrypsin[c]	293.2	8.05, 8.16, 8.10, 8.04	8	Hartley (1964)
Chymotrypsinogen	293.3	8.21, 8.60, 8.16, 8.80	8	Hartley (1964)
Thermolysin	292.5	2.91, 3.30, 3.30, 3.22, 3.21	3	Titani *et al.* (1972)
Deoxyribonuclease	293.2	2.90, 2.99, 2.82, 2.98, 2.82	3	Salnikow *et al.* (1970)
Carbonic anhydrase	292.5	6.91, 7.52, 7.55, 6.42, 7.92	7	Nyman and Lindskog (1964)
Subtilisin (Novo)[c]	292.4	2.68, 2.75, 2.72, 2.71	3	Matsubara *et al.* (1965)
Carboxypeptidase A	292.8	7.50, 7.40, 7.84, 7.94	7	Bradshaw *et al.* (1969)
Staphylococcus nuclease	293.0	1.12	1	Taniuchi *et al.* (1967)
DIP-Trypsin	293.0	3.91	4	Keil (1971)
Neutral protease	292.0	4.20	4	Tsuru *et al.* (1965)
Avidin	293.0	16.5	16	Green and Toms (1970)
Bovine serum albumin	293.3	1.82, 1.73	2	King and Spencer (1970)
Aldolase	292.5	11.6	12	Penhoet *et al.* (1969)
α-Lactalbumin	293.0	4.13	4	Brew *et al.* (1967)

[a] Measured in 0.05 M Tris (pH 7.5)–0.1 M NaCl. Protein concentrations were based upon published absorptivities. [b] Where multiple determinations are shown, analyses were performed on different occasions in the course of 22 months employing different preparations of the same protein. [c] For these determinations a single lot of lyophylized material was used. Measurements were made on freshly prepared samples over the period of 1 year.

At neutral pH the long wavelength limb of the negative MCD band of tyrosine centered at 275 nm partially overlaps the positive tryptophan band, resulting in an apparent decrease of intensity of the latter. The extent and significance of such overlap was estimated by examining the magnitude of the ellipticity of concentrated solutions of *N*-acetyl-L-tyrosine amide in the region near 293 nm (Table III). At 293 nm, the λ_{max} of the tryptophan band of most proteins, the rotation of *N*-acetyl-L-tyrosine amide (-3.8 deg cm^2 decimol^{-1} kG^{-1}) is 0.5% that at the λ_{max} of *N*-acetyl-L-tryptophan amide. The occurrence of the λ_{max} of proteins at a wavelength near 293 nm minimizes the correction needed for tyrosine overlap; however, the values of Table II are corrected for this overlap by summing the tyrosine contribution at the λ_{max} used for measurement of the tryptophan band using the values of Table III and the tyrosine content of the protein and subtracting this calculated value from the measured intensity of the tryptophan band. Such corrections are small in all proteins examined thus far, ranging from 18 to 112 deg cm^2 decimol^{-1} kG^{-1}. They become significant only when the ratio of tyrosine to tryptophan is very high, manifesting as an apparent reduction of the total tryptophan ellipticity. In no instance examined by us so far did these corrections exceed 5%.

In 6 M guanidine hydrochloride most proteins are randomly oriented and under these circumstances their tyrosyl and tryptophyl absorption is nearly identical with that of a solution containing equimolar residue concentrations of these amino acids (Tanford, 1970). We have compared the MCD of a number of proteins in water and in 6 M guanidine hydrochloride to assess the effect of protein conformation on the MCD spectra of tryptophan (Table IV). In native proteins the λ_{max} of the tryptophan band varies from 292.0 nm for *B. subtilis* neutral protease to 293.3 nm for bovine serum albumin. In 6 M guanidine hydrochloride the λ_{max} of all proteins examined thus far is 292.1 ± 0.1 nm, which is also the wavelength of maximal magnetic ellipticity of *N*-acetyl-L-tryptophan amide in this solvent. In 6 M guanidine hydrochloride, changes in the magnitude of tryptophan bands of proteins are generally small and do not exhibit characteristic trends.

Chemical substitution of the indole ring markedly alters the distinctive spectral features of this chromophore. The MCD of a series of such indole derivatives has been examined to assess the value of MCD in studying chemical modifications of tryptophyl residues.

The MCD spectrum of each derivative is unique (Figure 7). The spectra of *N*-acetylindole and 1-indolecarboxylic acid ethyl ester are similar to that of free tryptophan, with major positive bands at 289 and 292 nm, respectively, but their intensities are considerably lower. In contrast, 2-indolecarboxylic acid exhibits an intense positive MCD band at 305 nm (1180) and a negative band at 280 nm of similar magnetic molar ellipticity. Its absorption maximum is at 289 nm. The MCD effects of

TABLE III: Magnetic Molar Ellipticity of *N*-Acetyl-L-tyrosine Amide from 289 to 295 nm.[a]

λ (nm)	$[\theta]_M$[b]
289	−16.3
290	−11.6
291	−7.8
292	−5.5
293	−3.8
294	−2.8
295	−2.0

[a] Determined from the MCD of concentrated *N*-acetyl-L-tyrosine amide (pH 7.5) in 0.1 M NaCl –0.05 M Tris. [b] Average of two independent determinations.

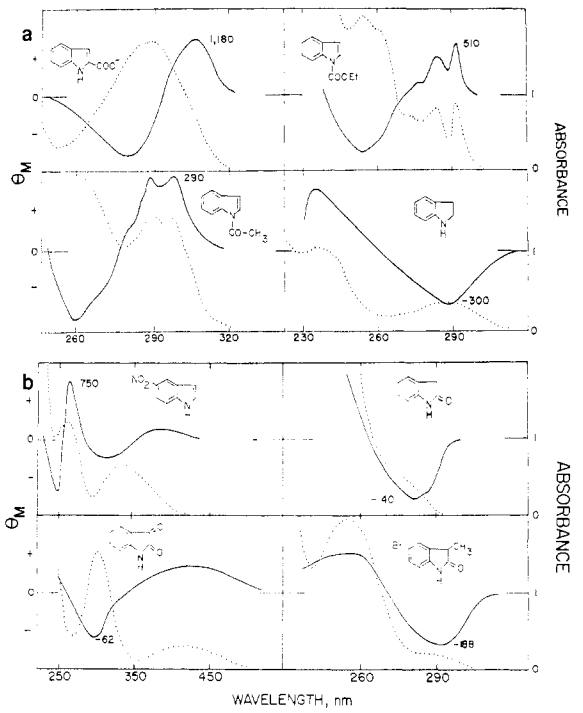

FIGURE 7: (a and b) MCD (—) and absorption (- - -) spectra of indole derivatives. Conditions: pH 7.5, 0.05 M Tris, 25°. Numbers adjacent to the major MCD bands are the molar magnetic ellipticities of that band in units of deg cm^2 decimol^{-1} kG^{-1}.

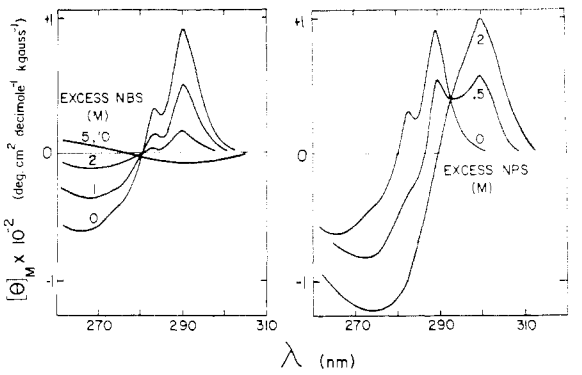

FIGURE 8: (Left) MCD in the course of the reaction of NBS with L-tryptophan. Appropriate aliquots of NBS, 2.5 × 10^{-2} M, in water were added to L-tryptophan, 2.54 × 10^{-4} M, dissolved in acetate buffer, 0.1 M (pH 5.0). MCD was examined both immediately after initiation of the reaction and subsequent to 10 min of incubation without apparent difference. (Right) MCD in the course of the reaction of NPS with L-tryptophan. NPS, dissolved in 97% formic acid, was added to L-tryptophan in 97% formic acid in the molar excessed shown and the mixture was allowed to react for 4 hr at room temperature. The solutions were then diluted with water to a residual formic acid concentration of 3% followed by recording of the MCD spectra.

5-nitroindole above 290 are minimal even though there is an absorption maximum near 330 nm. The magnetically induced ellipticity of isatin (2,3-indolinedione) which absorbs at 410 nm, is also weak with λ_{max} at 300 (−62) and 425 nm (+40). Other oxidized forms of indole, *e.g.*, 2-oxindole and 5-bromo-3-methyloxindole, which are similar to the products of the reaction of *N*-bromosuccinimide with tryptophan, exhibit spectra with negative bands centered at 280 (−140) and 290 nm (−188), respectively. Indoline, formed by reduction of the 1-2 double bond of indole, similarly exhibits negative elliptivity at 290 nm (−300).

MCD provides an effective means to follow the course of the reaction of free tryptophan either with *N*-bromosuccinimide (NBS) or with *o*-nitrophenylsulfenyl chloride (NPS) (Figure 8). Sequential addition of NBS to free tryptophan rapidly abolishes the 290-nm band. Beyond a fivefold molar excess of reagent, changes in the spectrum are no longer observed. The shape and intensity of the resulting spectrum is similar to that of the product of the reaction of NBS with skatole, *i.e.*, 5-bromo-3-methyloxindole.

In contrast, reaction of NPS with tryptophan results in the formation of the 2-substituted derivative, 2-(*o*-nitrophenylsulfenyl)tryptophan (Scoffone *et al.*, 1968). The substitution eliminates the 290-nm band and generates a new band at 303 nm with a magnetic ellipticity $[\theta]_M \cong 1000$, an intensity somewhat greater than that of the parent tryptophan band.

The reaction of NPS with lysozyme was studied under conditions previously shown to modify all six of its tryptophyl residues (Scoffone *et al.*, 1968). Incremental additions of NPS lead to the appearance of a new MCD band at 303 nm with concomitant loss of the positive peak at 293 nm. A 29-fold molar excess of NPS modifies all tryptophans resulting in a spectrum identical with that of the product of the reaction of excess NPS with *N*-acetyl-L-tryptophan amide. Based either on decreased ellipticity at 291 nm or on increased ellipticity at 303 nm, the number of tryptophans modified corresponds closely to that measured by absorption of the *o*-nitrophenyl chromophore.

TABLE IV: Effects of 6 M Guanidine Hydrochloride on the Tryptophan MCD of Proteins.[a]

	λ_{max} (±0.2 nm)		$[\theta]_M \times 10^{-3}$	
	Water	Guanidine	Water	Guanidine
Lysozyme	292.8	292.1	4.67	4.60
Chymotrypsin	293.2	292.0	6.28	6.35
Pepsin	292.6	292.1	3.81	4.23
Bovine serum albumin	293.3	292.0	1.35	1.27
Carbonic anhydrase	292.5	292.0	5.09	4.94
Thermolysin	292.5	292.1	2.57	2.37
Deoxyribonuclease	293.2	292.0	2.40	2.45

[a] Measured at 40 kG. Concentrated solutions of protein were made in 0.05 M Tris–0.1 M NaCl (pH 7.5). Just prior to analysis 1-ml aliquots of the protein were added to 5 ml of water and to 5 ml of 7.2 M guanidine hydrochloride. Values of $[\theta]_M$ are corrected for natural CD.

Discussion

In spite of the availability of convenient and accurate methods for the analysis of most amino acids, the determination of tryptophan has remained difficult. Largely owing to its acid lability, methods for its analysis place emphasis on hydrolysis under carefully controlled acidic (Liu and Chang, 1971; Gruen and Nicholls, 1972) or alkaline conditions in the presence of reducing agents (Hugli and Moore, 1972). Alternatively, spectrophotometric procedures employ reagents which produce colored products on reaction with tryptophan (Spies and Chambers, 1949; Barman and Koshland, 1967; Gaitonde and Dovey, 1970; Boccu *et al.*, 1970; Scoffone *et al.*, 1968).

The near-ultraviolet absorption spectrum of tryptophan in alkali (Goodwin and Morton, 1946) or in guanidine hydrochloride (Edelhoch, 1967) have also been employed. However, interference by the absorption bands of tyrosine or cysteine limits the accuracy of these latter procedures. The intense and unique MCD band of tryptophan near 290 nm (Figures 1 and 5) allows its direct and quantitative measurement with exceptional sensitivity and accuracy under conditions which preserve the integrity of the native protein structure and function. This results in a nondestructive, analytical procedure which permits either concomitant or subsequent measurements of protein function and structure on the same material.

Prior to establishing the tryptophan content of the native material, it is essential to determine the wavelength of maximal tryptophan ellipticity by scanning the tryptophan peak. For instance, the maximum has been found to vary from 292 nm for *B. subtilis* to 293.3 nm for bovine serum albumin and chymotrypsinogen. While at first glance this shift of 1.3 nm would appear to be minor, the marked change of intensity of the MCD band as a function of wavelength could introduce significant errors in quantitation. Hence, before final measurement, the location of the maximum must be determined for each protein. All data presented are based on this procedure (Tables I, II, and IV and Figure 6).

This positive MCD band exhibited by tryptophan is unique among all the amino acids. It can be differentiated clearly from all other magnetic effects since the λ_{max} near 293 nm is outside the region where the MCD for other amino acids is observed. As a consequence, measurements of both the location and intensity of the indole band between 290 and 293 nm are precise and largely free from interferences, allowing its unambiguous identification. The present data confirm our preliminary report (Holmquist, 1971) and are in accord with those of Barth *et al.* (1971).

We have here examined a number of well-characterized proteins, and the data establish that the intensity of the positive MCD band allows the direct measurement of tryptophan content (Figure 6). The proteins shown in Figure 6 were chosen for their purity, their molar absorptivities, and their tryptophan contents, determined from amino acid analysis and sequence data. Their tryptophan contents correlate with the magnitude of the 290–293-nm MCD peak. The molar magnetic ellipticity per tryptophyl residue of these proteins, derived from the slope of the data in Figure 6, is 777 deg cm^2 decimol^{-1} kG^{-1}. This value has served to quantitate the tryptophan content of a larger group of proteins with a variety of tryptophan contents. These values, as determined by MCD, also agree well with those of literature data (Table II).

For the majority of these proteins multiple determinations were performed at various times and employing different preparations of the same protein. Measurements over a period of 22 months on single lots of lysozyme, chymotrypsin, and subtilisin (Novo) were very reproducible, with deviations of 3.4, 2, and 1.5%, respectively. An estimated overall error based on the summation of operational factors involved in MCD, *i.e.*, signal/noise, magnetic field setting, etc., and adsorption measurements used for protein concentration determination, would approximate 5%, actually exceeding that encountered over this period of time.

The capacity to measure tryptophan by this technique benefits from the minimal overlap of its MCD band with those of phenylalanine and tryosine in the near-ultraviolet region (Figure 3). The overlap of the positive tryptophan band near 293 nm with the negative 275-nm band of tryrosine is small. As has been reported (Holmquist, 1971; Barth *et al.*, 1971), the degree of interference is a function of the tyrosine/tryptophan ratio which varies from 0.5 for lysozyme, chymotrypsin, and chymotrypsinogen to 9.4 for bovine serum albumin (Table II). The contribution of tyrosine at 293 nm does not exceed 5% in any case examined thus far.

The close correlation of the tryptophan contents of proteins, as determined by MCD, with those reported in the literature based on other methods, suggests that local three-dimensional structural factors do not significantly influence either the intensity or wavelength of maximal ellipticity of MCD tryptophan spectra in proteins. Yet, in view of the albeit small variation in their λ_{max}, it seemed imperative to detail the dependence of the MCD spectra upon solvent perturbation to anticipate potential exceptions from present experience.

The ultraviolet absorption spectra of tryptophan containing peptides and proteins have consistently revealed significant spectral effects dependent on their three-dimensional structure, particularly when examined by perturbation spectroscopy employing organic solvents and protein denaturants, *i.e.*, guanidine hydrochloride, urea, and sodium dodecyl sulfate (Bailey *et al.*, 1968; Donovan, 1969; Edelhoch, 1967). Such perturbation spectra have led to a differentiation between "exposed" or "buried" tyrosyl and tryptophyl residues based on their accessibility to solvents. Exposure of *N*-acetyl-L-tryptophan ethyl ester to solvent perturbants has served as a model for such investigations (Herskovits and Sorensen, 1968a).

We have examined the absorption spectrum of *N*-acetyl-L-tryptophan amide in identical fashion and with entirely analogous results. The MCD spectra of *N*-acetyl-L-tryptophan amide in the same organic solvents reflect similar systematic changes (Figure 2) indicating that these solvents also induce MCD spectral shifts, albeit of minor magnitude. These findings suggested analogous studies of tryptophan MCD in proteins using 6 M guanidine hydrochloride as a perturbant. This denaturant consistently shifts their maximal ellipticity to 292.1 nm to correspond with that of *N*-acetyl-L-tryptophan amide in this solvent, irrespective of the position of this band in the native protein. Hence, under these conditions the tryptophyl residues of these proteins apparently become spectrally equivalent, as has been observed previously by other methods, *e.g.*, lysozyme and chymotrypsin (Edelhoch, 1967). The results imply that the solvents completely abolish whatever structural features may account for the differences in the native proteins.

In principle it should then be possible to design a procedure for the quantitation of tryptophan content based on the measurement of MCD in 6 M guanidine hydrochloride at one wavelength, 292.1 nm. However, the present experiments demonstrate that any advantage gained by assessment of tryptophan content at a single wavelength, as would be possible by measurements in, *e.g.*, guanidine hydrochloride, is more than outweighed by the variability resulting from problems in handling and protein concentration determinations in such solvents.

The effects of N- and C-terminal substituents on the MCD of tryptophan were also examined to evaluate possible uncertainties arising from the presence of terminal tryptophyl residues. However, substitution of the amino or carboxyl group of tryptophan also results in but minor variations in band positions and intensities, though the existence of substitution can be detected (Table I), since it can vary λ_{max} from 290.5 to 291 nm, with magnetic molar ellipticities varying from 770 to 798 deg cm^2 decimol^{-1} kG^{-1}. The presence of a free α-amino group in L- or D-tryptophan and in L-tryptophan amide shifts the major positive band to 289.6 nm with a con-

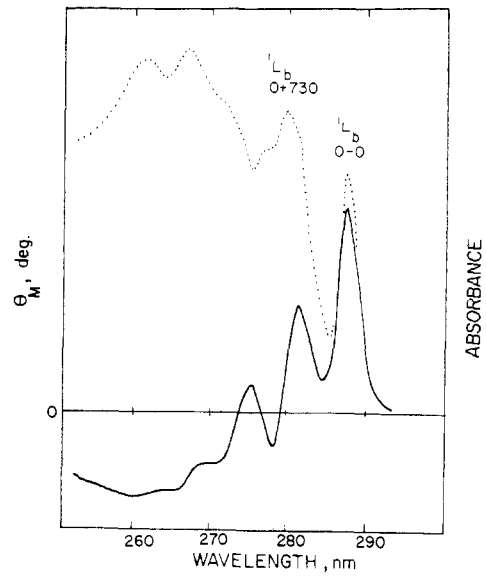

FIGURE 9: MCD (—) and absorption (- - -) spectra of indole in cyclohexane. The MCD spectrum was determined at 40 kG with the instrument programmed to a constant-spectral bandwidth of 1 nm.

comitant small increase in ellipticity (Table I). However, such variation in the analysis of protein tryptophan content is very likely to be insignificant.

The origin of the MCD bands of indole chromophores of proteins and of simple tryptophan derivatives has been identified by comparison with their absorption bands. The electronic transitions resulting in the adsorption spectra of indole and 3-methylindole have recently been detailed and assigned (Strickland *et al.*, 1970). In the region near 280 nm, the ^1L_a and ^1L_b transitions overlap and exhibit extensive fine structure associated with vibrational states. Figure 9 shows the absorption and MCD spectra of indole when dissolved in cyclohexane, a solvent affording high resolution of these spectral details. The absorption spectrum is virtually identical with that in methylcyclohexane (Strickland *et al.*, 1970). MCD spectra of indoles when dissolved in both water and hydrocarbons exhibit two major bands of opposite sign thought to be B terms based on considerations of symmetry (Shatz and McCaffery, 1969; Djerassi *et al.*, 1971). The absorption spectrum in cyclohexane exhibits the major ^1L_b band, the 0–0, as a peak at 287 nm and the 0 + 730 as a peak at 282 nm. They manifest as well resolved, positive bands in the MCD spectrum. Throughout the MCD spectrum the ^1L_a band is negative with vibrational fine structure. In water the positive bands in the MCD spectrum of indole appear at higher wavelengths, 284 and 290 nm.

The MCD spectra of tyrosine and its derivatives can be attributed to the ^1L_a band observed at 275 nm in their absorption spectra. Ionization of the phenol to the phenolate shifts the absorption maximum to 295 nm and reflects in the negative MCD trough at 295 nm, with twice the intensity of the 275 band (Figure 4). The ionization of tyrosyl residues in proteins, though not shown, is also reflected in a substantial component of negative ellipticity near 295 nm, thus limiting the pH for tryptophan measurements to the pH 8 and below where tyrosyl residues remain protonated.

The extreme intensity of the positive ^1L_b transition of the indole chromophore relative to that of the maximal natural CD is of major importance. In most proteins, the contribution of side-chain Cotton effects in the CD near 293 nm is small, generally representing no more than 5% of the corresponding MCD intensity at this wavelength. With the instrumentation used, the high intensity, 777 deg cm^2 decimol^{-1} kG^{-1} per tryptophan, allows reliable measurement of 10 nmol of this amino acid, which is equivalent to the sensitivity achieved by destructive tryptophan methods.

The assignment of the positive MCD bands of tryptophan to the ^1L_b transition bears upon its sensitivity to perturbants. Solvent perturbation of the absorption spectra of a number of proteins containing single tryptophyl residues demonstrate minimal variation of this band (Strickland *et al.*, 1971). Hence, theoretically, environmental factors would not be expected to extensively alter either the position or intensity of the 290-nm MCD tryptophan band in accord with experiment.

It has remained difficult to determine the tryptophan content of conjugated proteins when cofactors or prosthetic groups exhibiting magnetically induced ellipticity near 293 nm are present. The heme group, pyridoxal phosphate, and nonheme iron (Ulmer *et al.*, 1973) have all been found to interfere with tryptophan measurements by MCD. Qualitative identification, however, is possible owing to the shape and prominence of the 293-nm peak. Quantitation in such cases might be possible either by removal of the prosthetic group and measurements of the apoprotein or by measurement of the contribution of the isolated prosthetic group, experiments which remain to be completed.

Chemical modifications of specific side chain groups of amino acids commonly serve to assess the structural and functional properties of proteins. Tryptophan, a component of most proteins, stabilizes tertiary structure by interaction with other hydrophobic moieties and has been found essential to enzymatic catalysis in a number of instances (*e.g.*, Poulos and Price, 1971; Robinson, 1970). As shown here, MCD measurements are convenient to monitor the modification of tryptophyl residues, are capable of quantitating the number of tryptophan residues modified, and can even serve to identify the nature of the products formed.

Chemical modifications or substitutions of the indole ring markedly and characteristically alter the MCD spectrum (Figures 7 and 8). The reaction of NBS with tryptophan significantly decreases the 293-nm peak. This complex reaction yields a number of products, among them the 5-bromooxindole derivative of tryptophan (Witkop, 1961). Similarly, the major product of the reaction of skatole with NBS is 5-bromo-3-methyloxindole, the MCD spectrum of which has been determined (Figure 7). The replacement of the sharp positive band by a broad negative one near 293 nm seems characteristic of the oxidative process. Although the complexity of the reaction precludes detailed characterization of all reaction products by MCD at this time, it allows identification of the general nature of this reaction as an oxidation process, and application to other chemical modifications involving the oxidation of tryptophan such as photooxidation, ozonization, and iodination can be expected.

The chemical modification of both free tryptophan and lysozyme with *o*-nitrophenylsulfenyl chloride (Figure 8) which results in the loss of the 293-nm band of the starting material generates an even more intense positive band at higher wavelength. In this instance, both the disappearance of the substrate, tryptophan, and the appearance of product can be quantitated by MCD. In this particular modification the incorporation of the *o*-nitrophenolate moiety can also be quantitated spectrophotometrically (Scoffone *et al.*, 1968). The results obtained by both methods correlate well.

The potential of the MCD method for tryptophan quantitation and modification is high and its extension promises to be

of great value to many aspects of protein composition, structure, and function.

Acknowledgment

We are very much indebted to Dr. David D. Ulmer for his helpful advice and discussions in the course of this work.

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