Spectroscopic Determination of Tryptophan and Tyrosine in Proteins*

Harold Edelhoch

ABSTRACT: A rapid method for the determination of tryptophan in proteins is presented. It is based on absorbance measurements at 288 and 280 mμ of the protein dissolved in 6 M guanidine hydrochloride. Blocked tryptophanyl (*N*-acetyl-L-tryptophanamide) and tyrosyl (glycyl-L-tyrosylglycine) compounds were selected as model substances to represent the absorbance of the two chromophores in proteins. Tyrosine is measured simultaneously with tryptophan but with much less precision in proteins where most of the absorption is due to tryptophan. An independent procedure of tyrosine analysis is presented also which is based on its ionization in alkali.

Current methods of protein amino acid analysis do not give quantitative values for tryptophan and consequently the amino acid compositions, which are otherwise complete, fail to report tryptophan values. The principal reason for this situation is that the standard procedure of protein hydrolysis in strong acid results in the destruction of tryptophan (Hill, 1965). Therefore a second procedure is required to measure tryptophan. Alkaline hydrolysis is less destructive but does not give quantitative recoveries generally (Spies and Chambers, 1949). Enzymatic hydrolysis of proteins can give quantitative yields of tryptophan but this method may not be generally valid (Hill and Schmidt, 1962).

The hydrolytic problem can be circumvented by measuring tryptophan in the intact protein. A chemical method has been developed which has not been exploited adequately (Spies and Chambers, 1948, 1949). On the other hand, considerable effort has been expended in developing absorption spectroscopic procedures to measure tryptophan and tyrosine in unhydrolyzed proteins. Holiday (1936) and Goodwin and Morton (1946) have measured the absorption of proteins in 0.1 M NaOH and computed their tryptophan and tyrosine contents based on comparison with the absorption of the two amino acids. A modification of these techniques has been presented by Bencze and Schmid (1957). The preceding three methods do not give quantitative results. The behavior of the chromophores has not been normalized and the two models, *i.e.*, tryptophan and tyrosine, are not completely adequate. A procedure is suggested in this report which strongly reduces or eliminates these interactions, normalizes their absorption, and consequently permits a more precise analysis of tryptophan and tyrosine in proteins.

Materials

Low Molecular Weight Compounds. *N*-Acetyl-L- tryptophanamide was obtained from the Cyclo Chemical Corp. (R4739 CCP). It was listed as being chromatographically pure in two solvents. *Anal.* Calcd: N, 17.20. Found: N, 16.88. This compound was recrystallized two times in the author's laboratory. The first time it was recrystallized from acetone and the second time from ethanol. The molar absorption curve remained constant between 260 and 315 mμ after each crystallization.

Acetyl-DL-methionyl-L-tryptophan was a gift from E. Gross (National Institutes of Health, Bethesda, Md.). *N*-Acetyl-DL-tryptophan was a Sigma Chemical Co. product. It was a Σ grade compound (A99-074). L-Tryptophylglycine and acetyl-L-tyrosinamide were gifts from M. Wilchek (Weizmann Institute of Science, Rehovoth, Israel) and were chromatographically pure. L-Tryptophan was purchased from Nutritional Biochemical Corp. and Sigma Chemical Co. (Σ grade). L-Tyrosine was obtained from Fisher Scientific Co. and from Schwarz BioResearch Inc. (lot 6603). Glycyl-L-tyrosylglycine was a gift from H. Cahnmann and T. Shiba (National Institutes of Health, Bethesda, Md.) and was chromatographically pure. *N*-Acetyl-L-tyrosine ethyl ester was a product of Mann Research Laboratory (1188). L-Leucyl-L-tyrosine was obtained from the Nutritional Biochemical Co. L-Cystine was purchased from Mann Research Laboratory and Schwarz BioResearch, Inc. (6601).

Proteins. Trypsinogen (one time recrystallized 695B), lysozyme (two times recrystallized LYSF635), bovine pancreatic ribonuclease (RaF6096), and chymotrypsinogen (678-84A) were obtained from Worthington Biochemical Corp. Essentially identical results (tryptophan and tyrosine content) was obtained with chymotrypsinogen prepared by the Sigma Chemical Co. (six times recrystallized C32B-95) as with the Worthington sample. Horse heart apomyoglobin was a gift from A. Schechter (National Institutes of Health, Bethesda, Md.) and contained less than 1% heme.

* From the Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received February 27, 1967.*

Guanidine Hydrochloride. This compound is routinely referred to as guanidine hydrochloride. Most of the measurements were made with a product purchased from Eastman Organic Chemicals. To reduce its absorption in the near-ultraviolet region it was decolorized by treating with Norit for 1 hr at 45°. A precipitate formed at alkaline pH values. Concentrated solutions of the decolorized material were brought to pH 10.5 and filtered to eliminate the alkali-insoluble impurities.

Guanidine hydrochloride, purchased from the Aldrich Chemical Co. (Washington, D. C.), could be used directly since it showed none of the above-mentioned problems connected with its purity.

Methods

A Cary spectrophotometer Model 14 was employed to determine absorption spectra. A Beckman Model DU spectrophotometer was used for absorption measurements at specific wavelengths. pH measurements were made with a Radiometer TTT1 meter.

Basis of Method

The principal problem concerning the application of absorption spectroscopy to the analysis of tryptophan and tyrosine in proteins is the effect of the compact, globular form of most proteins on the absorption spectrum of the component amino acids which absorb in the near-ultraviolet region. The second problem is to select the appropriate model substances, the sum of whose absorption should agree with that of the protein.

The problem of the protein structure may be largely resolved by destroying the structure in a concentrated guanidine hydrochloride solution. Numerous studies have shown that proteins are highly unfolded and solvated in solutions of 5–6 M guanidine hydrochloride (Tanford *et al.*, 1966, 1967; Kielley and Harrington, 1960; Buckley *et al.*, 1963; Kawahara *et al.*, 1965). In choosing a model compound for the tryptophanyl chromophore in proteins, the effect of neighboring substituents must be considered. The absorption spectrum of tryptophan is much more influenced by the charged state of the amino than that of the carboxyl group (Donovan *et al.*, 1961). Since the tryptophanyl chromophore in a protein will usually have two neighboring peptide groups, a tryptophan derivative whose α-amino and α-carboxyl groups are blocked by peptide linkages should be a suitable model. The α-amino group has almost no effect on the absorption of the tryptophanyl residue when it is separated from the indole chromophore by a glycyl residue as in glycyltryptophan (Donovan *et al.*, 1961).

In nonconjugated proteins the amino acid tryptophan absorbs at the longest wavelengths. Above 295 mμ the absorption of the protein is essentially determined by its tryptophan content (Wetlaufer, 1962). However the tryptophan absorption spectrum changes rapidly in this wavelength region, and it is more advisable to make observations near its two absorption peaks and correct for the contributions of other residues. Only cystine and tyrosine absorb significantly, with the latter amino acid making the more important contribution (Wetlaufer, 1962; Beaven and Holiday, 1952). If the cystine content is known its contribution can be accounted for. It is then possible by making measurements at two wavelengths and solving the simultaneous equations to determine the content of tyrosine and tryptophan. Since the absorption of the latter is about four times the former residue, at their respective peaks, the precision in the determination of tryptophan will greatly exceed that of tyrosine. For this reason an independent method of tyrosine analysis is also presented.

Results

Simultaneous Tryptophan and Tyrosine Analysis. LOW MOLECULAR WEIGHT MODEL COMPOUNDS. There is a shift toward the red of about 1.0 mμ in the wavelength of the absorption peak of the nitrogen-blocked tryptophan compounds, *i.e.*, *N*-acetyl-L-tryptophanamide, acetyl-DL-methionyl-L-tryptophan, and *N*-acetyl-L-tryptophan, compared to the compounds with a free α-amino group, as in tryptophan and L-tryptophylglycine (Table I).

TABLE I: Absorption Properties of Tryptophanyl Derivatives in 6.0 M Guanidine Hydrochloride (pH 6.5)–0.02 M Phosphate.

Buffer	Major Peak (mμ)	ϵ	Minor Peak (mμ)	ϵ
N-Acetyl-L-tryptophanamide	280.8	5690	289.0	4850
N-Acetyl-L-tryptophan	281.0	5500	289.2	4700
Acetyl-DL-methionyl-L-tryptophan	281.0	5650	289.3	4810
L-Tryptophan				
Sigma	280.0	5800	288.5	4800
Nutritional	280.0	5800	288.5	4730
L-Tryptophylglycine	279.8	5650	288.5	4700

Beaven and Holiday (1952) have reported similar results. The spectral properties of the three nitrogen-blocked tryptophan compounds were similar with respect to wavelength peaks and molar extinction coefficients, although two of the compounds have their carboxyl groups free. Evidently substitution of the α-carboxyl position has relatively little influence on the absorption properties of the indole chromophore. This result is apparent also in the spectra of tryptophan and L-tryptophylglycine, which agree closely. The agreement in molar extinction coefficients of the five tryptophan derivatives listed in Table I suggests that the spectral properties of the indole chromophore are only slightly influenced by substituents. Of the various model compounds shown in Table I, *N*-acetyl-L-tryptophanamide

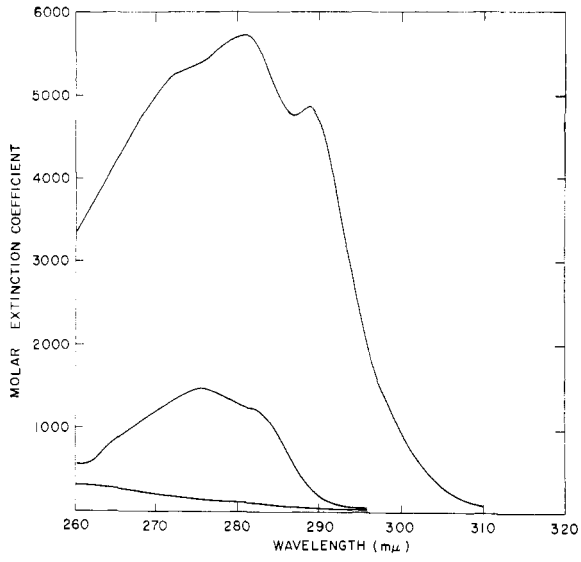

FIGURE 1: Absorption curves of *N*-acetyl-DL-tryptophanamide (upper), glycyl-L-tyrosylglycine (middle), and cystine (lower) in 6.0 M guanidine hydrochloride (pH 6.5)–0.02 phosphate buffer.

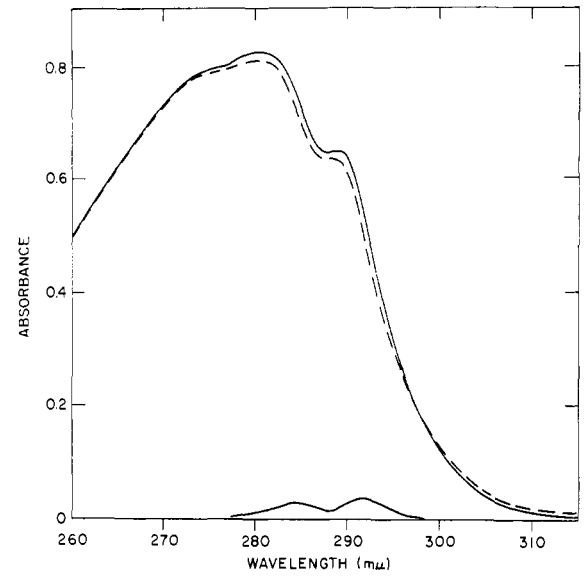

FIGURE 2: Comparison of absorption spectrum of chymotrypsinogen (solid line) (1.61×10^{-5} M) with solution of model compounds containing equiresidue concentrations of *N*-acetyl-DL-tryptophanamide, glycyl-L-tyrosylglycine, and cystine in 6.0 M guanidine hydrochloride (pH 6.5)–0.02 M phosphate buffer. (insert) Absorption difference spectrum between protein and model solution.

most closely resembles the structure of tryptophan in proteins. It was ascertained that this compound was pure by recrystallizing it from acetone and then from ethyl alcohol. No changes in absorption properties were observed after each recrystallization. Its spectrum is shown in Figure 1.

In contrast to the tryptophan derivatives, peptide substitution at the α-amino group of tyrosine had very little effect on the absorption properties of the phenolic chromophore. The molar extinction coefficient at the absorption peak was essentially constant in the four tyrosyl derivatives listed in Table II. The compound Gly-L-Tyr-Gly was selected as the most suitable model to represent the behavior of tyrosyl residues in proteins, and its spectrum is shown in Figure 1.

The only other α-amino acid found in nonconjugated proteins that absorbs above ~270 mμ is cystine. How-

TABLE II: Absorption Properties of Tyrosyl Derivatives in 6.0 M Guanidine Hydrochloride (pH 6.5).

	Peak (mμ)	ε
Gly-L-Tyr-Gly	275.5	1470[a]
Acetyl-L-tyrosinamide	275.5	1490
N-Acetyl-L-tyrosyl ethyl ester	275.5	1500[a]
Tyrosine		
Fisher	275.5	1475[a]
Schwarz	275.5	1515
L-Leucyl-L-tyrosine	275.5	1500

[a] Solutions contained 0.02 M phosphate.

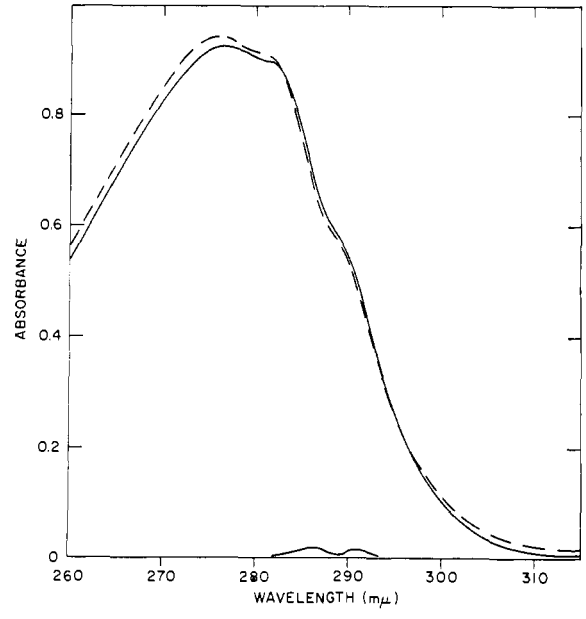

FIGURE 3: Comparison of absorption spectrum of trypsinogen (2.50×10^{-5} M) (solid line) with solution of model compounds containing equiresidue concentrations of *N*-acetyl-DL-tryptophanamide, glycyl-L-tyrosylglycine, and cystine in 6.0 M guanidine hydrochloride (pH 6.5)–0.02 M phosphate buffer. (insert) Absorption difference spectrum between protein and model solution.

TABLE III: Extinction Coefficient of Model Proteins.

	Mol Wt	λ (mμ)	E (1%)	Ref
Trypsinogen	24,248	280	13.9	Davie and Neurath (1955)
Chymotrypsinogen	25,670	282	20.0	Wilcox *et al.* (1957)
Lysozyme	14,307	280	26.5	Steiner (1964)
Horse heart apomyoglobin	17,000	280	8.4	Crumpton and Polson (1965)
Ribonuclease	13,680	280	6.95	Sherwood and Potts (1965)

ever its molar extinction coefficient at 280 mμ is only about 10% that of tyrosine and 2% that of tryptophan. The absorption spectrum of cystine is shown also in Figure 1.

PROTEIN MODELS. Four tryptophan-containing proteins (lysozyme, chymotrypsinogen, trypsinogen, and horse heart apomyoglobin) and one protein containing only tyrosine (ribonuclease) were chosen in order to compare their absorption with that of a solution consisting of equiresidue concentrations of the model compounds. These five proteins were selected for comparison because they are available in highly purified form, their composition and molecular weights are well established, and their extinction coefficients are known. Their pertinent properties are listed in Table III. Protein concentrations were determined from their extinction coefficients

In Figures 2–5 are shown the near-ultraviolet spectra of four of the above-listed proteins in 6.0 M guanidine hydrochloride (pH 6.5) and of solutions containing equiresidue amounts of *N*-acetyl-L-tryptophanamide, Gly-L-Tyr-Gly, and cystine in the same solvent. The spectra of the solutions containing the model substances closely resemble those of the protein except that the latter are shifted slightly to the red in all four cases. If the difference in absorption between the tryptophan-containing proteins and the model solutions is plotted, maxima are found at about 292 and 284 mμ. Numerous studies have shown that tryptophan compounds show characteristic difference peaks at these wavelengths (Bigelow and Geschwind, 1960; Chervenka, 1959; Yanari and Bovey, 1960; Foss, 1961). The origin of the difference spectra in the proteins is not clear. They may arise (1) from the existence of residual structure in the

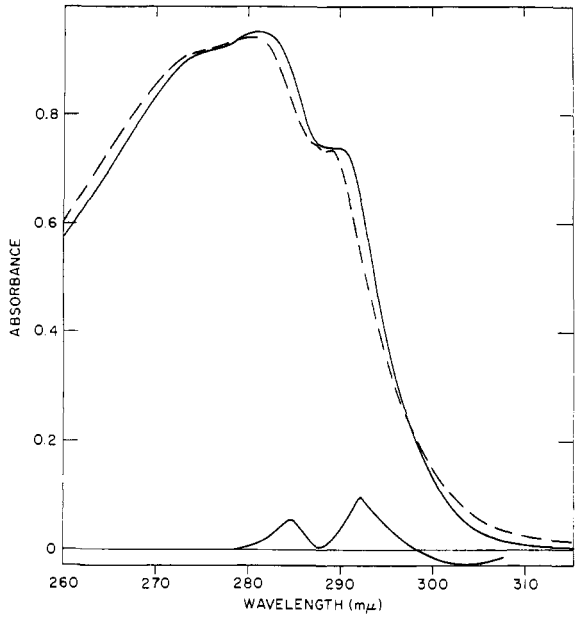

FIGURE 4: Comparison of absorption spectrum of lysozyme (2.44×10^{-5} M) (solid line) with solution of model compounds containing equiresidue concentrations of *N*-acetyl-DL-tryptophanamide, glycyl-L-tyrosylglycine, and cystine in 6.0 M guanidine hydrochloride (pH 6.5)–0.02 M phosphate buffer. (insert) Absorption difference spectrum between protein and model solution.

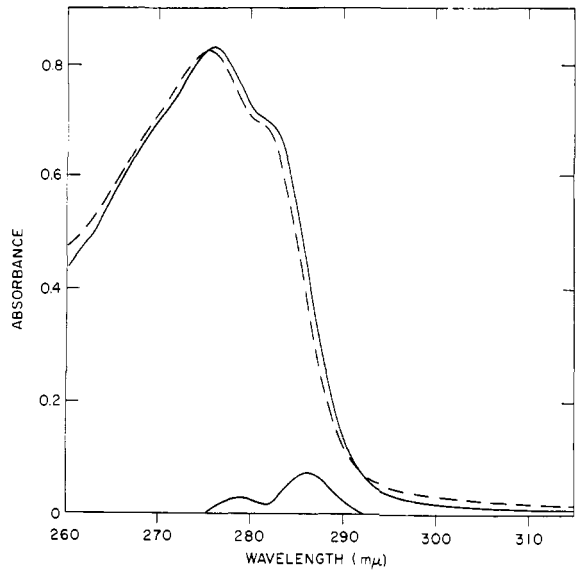

FIGURE 5: Comparison of absorption spectrum of ribonuclease (0.85×10^{-4} M) (solid line) with solution of model compounds containing equiresidue concentrations of glycyl-L-tyrosylglycine and cystine in 6.0 M guanidine hydrochloride (pH 6.5)–0.02 M phosphate buffer. (insert) Absorption difference spectrum between protein and model solution.

TABLE IV: Molar Extinction Coefficients of Model Substance in 6.0 M Guanidine Hydrochloride (pH 6.5)–0.02 M Phosphate Buffer.

	288 mμ	280 mμ	275.5 mμ
N-Acetyl-L-tryptophanamide	4815	5690	
Gly-L-Tyr-Gly	385	1280	1500
Cystine			
Mann	75	120	155
Schwarz	70	120	135

TABLE V: Tryptophan and Tyrosine Composition as Measured by Absorption Spectroscopy in 6.0 M Guanidine Hydrochloride Solutions (pH 6.5).

		Calcd		Found	
	M × 10^{5}[a]	Trp	Tyr	Trp	Tyr
Chymotrypsinogen	1.61	8	4	8.0	3.85
Trypsinogen	2.50	4	10	4.15	9.50
Lysozyme	2.44	6	3	5.95	3.55
Horse heart apomyoglobin	6.10	2	2	1.95	2.05
Ribonuclease	8.50		6		6.05

[a] Molarity of protein in cuvet in which absorbancies were measured.

protein resulting in incomplete solvation of the chromophores, (2) because the model substance may not be entirely satisfactory, or (3) because of other unknown properties. A similar situation prevails with ribonuclease. Difference peak occur at 286 and 279 mμ which are characteristic of the phenolic chromophore (Wetlaufer, 1956; Bigelow and Geschwind, 1960; Yanari and Bovey, 1960).

The tryptophan content of a protein may be evaluated from absorption measurements at wavelengths where tyrosine and cystine absorption is trivial, *i.e.*, above 295 mμ. Since the absorbance of tryptophan compounds changes rapidly with wavelength above 295 mμ a better procedure is to determine absorbance values near the tryptophan peaks. Minima in the difference spectra between the tryptophan-containing proteins and their model solutions occur near 288 and 280 mμ, wavelengths close to the two absorption peaks of *N*-acetyl-L-tryptophanamide (Table I). Consequently these two wavelengths have been selected. In Table IV are listed the molar absorption coefficients of the three model compounds at these wavelengths.

The tryptophan content of a protein can be determined from its absorption at 288 mμ if the tyrosine and cystine content are known from amino acid analysis and their absorption accounted for. The combined molar extinction coefficients of cystine and Gly-L-Tyr-Gly is less than 10% of that of *N*-acetyl-L-tryptophanamide at this wavelength (Table IV). If the cystine concentration is known then the concentration of tyrosine and tryptophan can be calculated from absorbance measurements at 288 and 280 mμ and the molar extinction coefficients listed in Table IV, by solution of the two simultaneous equations: $\epsilon_{288} = N_{Trp}4815 + M_{Tyr}385$ and $\epsilon_{280} = N_{Trp}5690 + M_{Tyr}1280$, where N and M are the numbers of moles of tryptophan and tyrosine per mole of protein; consequently $N_{Trp} = (\epsilon_{288}/3103) - (\epsilon_{280}/10{,}318)$. The concentration of tryptophan and tyrosine in the four model proteins has been calculated after subtracting from the absorbance values the calculated contribution of the cystine residues. These results are summarized in Table V. The tryptophan values agree better with the known values than do the tyrosine.

If the protein does not contain any tryptophan, as in ribonuclease, its absorption above ~270 mμ is determined by its content of tyrosine and cystine. The absorption spectrum of ribonuclease in 6.0 M guanidine hydrochloride at pH 6.5 was shifted slightly to the red compared to its model solution (Figure 5). However the curves cross at 275.5 mμ. A value of 6.05 moles of tyrosine/mole of ribonuclease has been computed (Table V) from its absorption at 275.5 mμ using values of 1500 and 145 for the molar extinction coefficients of the tyrosyl and cysteinyl residues. The latter are based on the molar extinction coefficients of Gly-L-Tyr-Gly and cystine, respectively, at 275.5 mμ in 6.0 M guanidine hydrochloride (Table IV).

Tyrosine Analysis. In proteins where tryptophan accounts for the major part of the near-ultraviolet absorption its concentration can be determined more precisely than that of tyrosine by absorption measurements. An independent method of tyrosine analysis, however, is available based on the major enhancement and shift toward longer wavelengths of its absorption spectrum that occurs with ionization (Crammer and Neuberger, 1943). Since the indole moiety does not have any ionizable groups its spectrum should be unaffected by the change in pH required to titrate the phenolic moiety. The dissociation curves of tyrosine and three tyrosyl peptides are shown in Figure 6. The pK of the phenolic group in 6.0 M guanidine hydrochloride is near 10.0 in all four compounds and does not appear to be significantly affected by neighboring residues. The change in molar extinction coefficient at two wavelengths near the absorption peak of the ionized species, *i.e.*, 295 and 300 mμ, is given in Table VI. It is seen that the three tyrosyl peptides have quite similar values.

Guanidine hydrochloride solutions (6 M) of the four proteins listed in Table VII were adjusted rapidly from pH 6.5 to ~12.5 with a small volume of 4 M KOH. The change in absorbance at 295 and 300 mμ was followed with time. A very small linear increase in absorbance was observed with time (~0.005/min) with chymotrypsinogen, lysozyme, and ribonuclease. The latter presumably

TABLE VI: The Change in Molar Extinction Coefficient with Ionization of Tyrosyl Derivatives in 6.0 M Guanidine Hydrochloride.

	Δε 295 mμ	Δε 300 mμ
Gly-L-Tyr-Gly	2470	2240
Acetyl-L-tyrosinamide	2520	2290
L-Leucyl-L-tyrosine	2445	2280
Average	2480	2270
Tyrosine	2590	2400

TABLE VII: Tyrosine Composition as Measured by Absorption Spectroscopy in 6.0 M Guanidine Hydrochloride Solutions at pH 6.5 and 12.5.

		Found	
	Calcd	295 mμ	300 mμ
Chymotrypsinogen	4	4.05	4.25
Lysozyme	3	2.80	3.35
Horse heart apomyoglobin	2	2.05	2.10
Ribonuclease	6	6.00	6.20

arise from minor turbidity effects in alkali. Measurements should, therefore, be made within 1 min after mixing or observations should be extrapolated to zero time of mixing. The tyrosine content was computed based on molar extinction coefficients of 2480 at 295 mμ and of 2270 at 300 mμ. It is seen that the values obtained at 295 and 300 mμ agree with each other in all cases except lysozyme. The low value at 295 mμ may result from a superimposed blue shift in the tryptophanyl spectrum if further structural disorganization occurs in alkali (Steiner, 1964). It should be noted that among the proteins studied the largest difference spectrum was obtained between lysozyme and its model system in 6 M guanidine hydrochloride at neutral pH (Figure 4). The tryptophanyl absorption is much less at 300 than 295 mμ and should give rise to a smaller difference spectrum. Protein structural changes which affect tryptophan absorption may be investigated in this way. Measurements at several wavelengths, *i.e.*, between 290 and 310 mμ, would enhance the sensitivity of this procedure.

It is seen from Table VII that reasonable values are obtained for the tyrosine content of the four proteins although they all appear to be slightly high. Trypsinogen could not be studied by this procedure since it was not soluble in 6 M guanidine hydrochloride at pH 12.5.

Discussion

It is clear from recent studies on the denaturation of proteins that the absorption spectra of tryptophanyl and tyrosyl residues are shifted slightly from their values in aqueous solution when they are either buried in the interior or lying on the surface of the protein (Wetlaufer, 1962). Most globular proteins show "denaturation blue shifts" when their native structures are modified by denaturing conditions, such as certain organic solvents and solutes, strong acid and base, high temperatures, etc. (Chervenka, 1960; Foss, 1961; Donovan *et al.*, 1961; Hermans and Scheraga, 1961a,b). Thus it is not possible to reproduce the absorption properties of the aromatic chromophoric groups in a protein since each residue not fully exposed to the solvent will have its spectrum more or less displaced from that in water. In order to circumvent this problem it is necessary to destroy the organized, *i.e.*, secondary, tertiary, structure of the protein. Aside from hydrolytic procedures the most effective denaturing reagent available at present is guanidine hydrochloride. Beaven and Holiday (1952) proposed making measurements in 0.1 M alkali in order to displace the spectrum of the tyrosyl group. Wetlaufer (1962) has suggested that a solution of 8 M urea in 0.1 M NaOH might be used to normalize the absorption properties of the chromophoric residues. Strong alkaline conditions, however, lead to the scission of disulfide bonds and occasionally to turbidity problems. Guanidine is a superior denaturant to urea (Edelhoch, 1958; Buckley *et al.*, 1963; Steiner, 1964; Kawahara *et al.*, 1965; Edelhoch and Steiner, 1966) and should normalize all the residues. Nevertheless, one cannot be absolutely sure that all the residues of a protein dissolved in 6 M guanidine hydrochloride have the same degree of solvation as they have in their amino acid form or in simple polypeptides. However, in spite of the imperfect fit of the spectra of the model solutions to those of the proteins, meaningful values can be obtained by using wavelengths where the small red shifts in the protein have their least influence. These have been found to occur at 280 and 288 mμ for the model proteins. Good agreement has been found for the tryptophan content of four proteins using absorption values of *N*-acetyl-L-tryptophan-

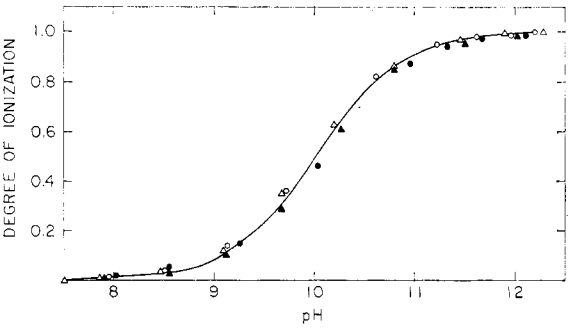

FIGURE 6: The ionization of tyrosine (●), acetyl-L-tyrosinamide (△), L-leucyl-L-tyrosine (▲), and glycyl-L-tyrosylglycine (○) in 6.0 M guanidine hydrochloride. The line is a theoretical dissociation curve with a p*K* of 10.02. Absorbance measured at 300 mμ.

amide in 6.0 M guanidine hydrochloride solution. Less satisfactory agreement was obtained for tyrosine from the absorbance values of the proteins in 6 M guanidine hydrochloride at neutral pH. However, the tyrosine values could be checked by a second method based on the ionization of the tyrosyl groups. This method should work better for proteins with high tryptophan to tyrosine ratios.

Beaven and Holiday (1952) and Wetlaufer (1962) have reviewed the application of spectroscopy to tyrosine and tryptophan analysis in proteins. The former reported that significant blue shifts occur in the spectra of proteins when they are digested by proteolytic enzymes. This method of eliminating the spectral shifts owing to protein interactions has not been evaluated since native proteins display variable susceptibility to individual proteolytic enzymes and considerable incubation periods are needed. The use of enzyme mixtures which lead to extensive digestion may result in both free and blocked α-aminotryptophan peptides and render questionable the model compounds that are chosen. Furthermore, products of digestion may not be soluble in aqueous solution. In addition to the superior solution properties of guanidine hydrochloride for proteins and the saving in time, ultraviolet-absorbing substances, which are noncovalently bound to the protein, may be removed by dialyzing the guanidine hydrochloride solution.

It should be stressed that anything which contributes to the absorbance at 280 or 288 mμ, whether a contaminant, a cofactor, or turbidity, will interfere with the spectral analysis. A good method of checking for absorbing impurities (or opalescence) is to measure the absorbance of the protein in 6 M guanidine hydrochloride at 315 mμ at neutral pH where the absorption of tyrosine and tryptophan is nil.

Acknowledgment

The authors wish to thank Mr. R. E. Lippoldt for valuable technical assistance.

References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 320.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.

Bigelow, C. C., and Geschwind, I. I. (1960), *Compt. Rend. Lab. Carlsberg 30*, 21.

Buckley, C. E., III, Whitney, P. L., and Tanford, C. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 827.

Chervenka, C. H. (1959), *Biochim. Biophys. Acta 31*, 85.

Chervenka, C. H. (1960), *J. Am. Chem. Soc. 82*, 585.

Crammer, J. L., and Neuberger, A. (1943), *Biochem. J. 37*, 302.

Crumpton, M. J., and Polson, A. (1965), *J. Mol. Biol. 11*, 722.

Davie, E. W., and Neurath, H. (1955), *J. Biol. Chem. 212*, 515.

Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 2686.

Edelhoch, H. (1958), *J. Am. Chem. Soc. 80*, 6648.

Edelhoch, H., and Steiner, R. F. (1966), *Biopolymers 4*, 999.

Foss, J. G. (1961), *Biochim. Biophys. Acta 47*, 569.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Hermans, J., Jr., and Scheraga, H. A. (1961a), *J. Am. Chem. Soc. 83*, 3283.

Hermans, J., Jr., and Scheraga, H. A. (1961b), *J. Am. Chem. Soc. 83*, 3293.

Hill, R. L. (1965), *Advan. Protein Chem. 20*, 37.

Hill, R. L., and Schmidt, W. R. (1962), *J. Biol. Chem. 237*, 389.

Holiday, E. R. (1936), *Biochem. J. 30*, 1795.

Kawahara, K., Kirshner, A. G., and Tanford, C. (1965), *Biochemistry 4*, 1203.

Kielley W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.

Sherwood, L. M., and Potts, J. T., Jr. (1965), *J. Biol. Chem. 240*, 3799.

Spies, J. R., and Chambers, D. C. (1948), *Anal. Chem. 20*, 30.

Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.

Steiner, R. F. (1964), *Biochim. Biophys. Acta 79*, 51.

Tanford, C., Kawahara, K., and Lapanje, S. (1966), *J. Biol. Chem. 241*, 1921.

Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Am. Chem. Soc. 89*, 729.

Wetlaufer, D. B. (1956), *Compt. Rend. Lab. Carlsberg Ser. Chim. 30*, 135.

Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.

Wilcox, P. E., Cohen, E., and Tan, W. (1957), *J. Biol. Chem. 228*, 999.

Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.