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The Reaction of Diazonium-1H-tetrazole with Proteins. Determination of Tyrosine and Histidine Content*

Mordechai Sokolovsky† and Bert L. Vallee

ABSTRACT: Coupling of diazonium-1H-tetrazole (DHT) with histidine and tyrosine and quantitative conversion to the respective bisazo derivatives serve as the basis of a method for the determination of these residues in proteins. This reagent, previously proposed for the determination of reactive histidyl residues (Horinishi, H., Hachimori, Y., Hurihara, K., and Shibata, K. (1964), *Biochim. Biophys. Acta 86*, 477), forms monoazo- and bisazotyrosine and -histidine derivatives each of which exhibits characteristic spectra. These differ to some extent from those of the respective *N*-acylamino acids and peptides. Their wavelengths of maximal absorption and molar absorptivities have been characterized and assigned and serve as the basis for the quantitative method proposed. The spectral characteristics of the peptides or acylamino acids both of tyrosine and of histidine have been found to be the proper standards of reference for the determination of the respective residues in proteins.

The reactivity of the proteins and these spectral characteristics of the derivatives combine to render results obtained on native proteins ambiguous: three of the possible azo derivatives, *i.e.*, mono- and bisazotyrosine and bisazohistidine, all absorb at 480 mμ, the wavelength previously employed for the measurement of histidine alone. Complete conversion at high DHT concentration of both monoazo to the respective bisazo species, combined with alkali denaturation of proteins prior to coupling, leads to a quantitative method for the simultaneous determination of the tyrosine and histidine contents of proteins by photometry at two wavelengths.

Diazonium compounds have been employed extensively to modify proteins, to study composition and structure (Howard and Wild, 1957; Higgins and Harrington, 1959; Tabachnick and Sobotka, 1960) and their relationship to function of enzymes (Fraenkel-Conrat *et al.*, 1949; Gundlach *et al.*, 1962), and also to produce specific antigenic determinants (Landsteiner, 1945). Diazonium compounds couple readily with histidyl, tyrosyl, and lysyl residues of proteins, but both the lack of specificity and the incomplete resolution of the spectral bands which accompany the formation of different azo derivatives of amino acids have restricted interest in their use as site-specific reagents.

Recently, Horinishi *et al.* (1964) have examined diazonium-1H-tetrazole[1] and proposed its use as a coupling reagent for proteins. The absorption bands of monoazotyrosine, bisazohistidine, and bisazotyro-

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[1] Abbreviation used: DHT, diazonium-1H-tetrazole.

sine,[2] with maxima at 380, 480, and 550 mμ, respectively, have been stated to be separate and distinct.

In the course of our studies, designed to delineate reactive amino acid side chains involved in the catalytic function of carboxypeptidase A, DHT has been employed for chemical modification. The spectral properties of the azotetrazole derivatives of histidyl and tyrosyl residues appeared more complex than had been anticipated and led to the present reinvestigation.

Materials

5-Amino-1H-tetrazole monohydrate, mp 197–210 dec (lit. 203° dec (Hantzsch and Vogt, 1901)), was obtained from the Aldrich Chemical Corp., Milwaukee, and from the Tokyo Chemical Industry, Japan. *N,O*-Diacetyl-L-tyrosine (mp 166–168°) and *N*-acetyl-L-histidine (mp 158° dec) were obtained from the Cyclo Chemical Corp., Los Angeles. *N*-Acetyl-DL-tyrosine (mp 85–88°), *N*-benzoyl-DL-tyrosine (mp 194–196°), *N*-carbobenzoxy-L-tyrosine (mp 95–105°), *N*-acetyl-L-tyrosine amide (mp 225–226°), *N*-acetyl-L-tyrosine ethyl ester (mp 79–80°), and glycyl-L-tyrosine amide were obtained from Mann Research Laboratories, New York. All compounds were chromatographically pure. α-Chymotrypsin, ovalbumin, ribonuclease, pepsin, trypsin (Worthington), and bovine insulin (Lilly) were all used without further purification. Polypeptides containing histidine and tyrosine were a gift of Dr. R. B. Riniker of CIBA, Basle (Switzerland). The concentrations of stock solutions of the peptides were determined by amino acid analysis after acid hydrolysis, using the method of Spackman *et al.* (1958).

Absorption Spectrophotometry. A Zeiss PMQII spectrophotometer was used for absorbance measurements at single wavelengths, and continuous spectra were obtained with a Cary Model 15 MS automatic recording spectrophotometer.

pH was determined with a Radiometer pH meter (Model pH M 4) equipped with a Radiometer GK 2021 electrode.

5-Diazo-1H-tetrazole was prepared by diazotization, in an ice bath, of 1 g of 5-amino-1H-tetrazole, dissolved in 23 ml of 1.6 M HCl with 0.7 g of sodium nitrite solution which was added slowly with continuous stirring. The diazo derivative was formed within 6–8 min with a yield of 75–95%, as determined by reaction with excess *N*-acetyltyrosine amide (*vide infra*). Since the diazo derivative decomposed in acidic solution, the mixture was adjusted to pH 5 after 6–8 min, and then diluted to the desired volume with cold water. Under these conditions diazotetrazole is stable for at least 1 hr at 0°.

It must be emphasized that concentrated solutions of diazotetrazole are highly explosive. The reagent must therefore be handled with extreme care and only in dilute solution. Solutions above 0.2 M should not be employed.

[2] Monoazo and bisazo refer to the 5-azo-1H-tetrazole derivatives of histidyl and tyrosyl residues.

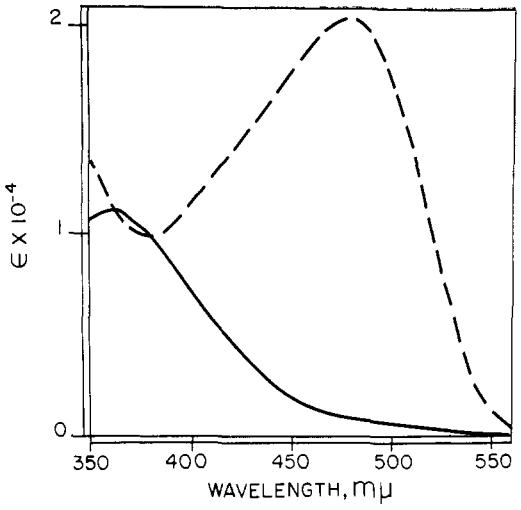

FIGURE 1: Absorption spectra of monoazo-*N*-acetylhistidine (——) and bisazo-*N*-acetylhistidine (---), bicarbonate buffer, pH 8.8. The derivatives were obtained by treating 1×10^{-4} M *N*-acetylhistidine with 1.2×10^{-4} M DHT and with 3×10^{-2} M DHT, respectively, 30 min, room temperature.

Preparation of N-Carbobenzoxy-3-monoazotetrazole-L-tyrosine. *N*-Carbobenzoxy-L-tyrosine, 315 mg (1 mM), was dissolved in 50 ml of 1 M $KHCO_3$ at pH 8.8 and 0.9 mM of DHT, pH 5.0, was added at 0°. The pH was adjusted to 8.8 with 1 N NaOH. The reaction mixture was kept at 0° for 1 hr, then removed from the ice bath, and kept at room temperature. After 30 min the pH was adjusted to 2 with HCl. The resulting precipitate was collected by filtration and washed several times with ethyl acetate to remove the unreacted tyrosine derivative, followed by a dilute ethanol wash. The orange-brown compound (yield 80%) was dissolved in water by raising the pH to 9 and precipitated by acidification. This process was carried out twice. After crystallization from methanol–water the melting point was 190–195°. *Anal.* Calcd for $C_{18}H_{17}N_7O_5 \cdot H_2O$: C, 50.3; H, 4.4; N, 22.8. Found: C, 50.2; H, 4.4; N, 21.8.

The concentration of DHT was determined by measuring the formation of *N*-acylmonoazotyrosine, rather than by using the reaction of the reagent with free histidine (Horinishi *et al.*, 1964). The histidine derivative exhibits maximal absorption between 360 and 380 mμ (*vide infra*) where DHT absorption is quite intense, and free amino acids have been found unsuitable for purposes of standardization (*vide infra*). Synthetic monoazo-*N*-carbobenzoxytyrosine, which absorbs maximally at 478 mμ, was used as a standard, since at this wavelength DHT exhibits minimal absorbance.

DHT solution (1 ml), approximately 1×10^{-3} M, was mixed with 9 ml of 5×10^{-2}–3×10^{-3} M *N*-acetyltyrosine or *N*-acetyltyrosine amide, pH 8.8. The mixture was kept at room temperature for 20–30 min. Under these conditions DHT reacts stoichiometrically

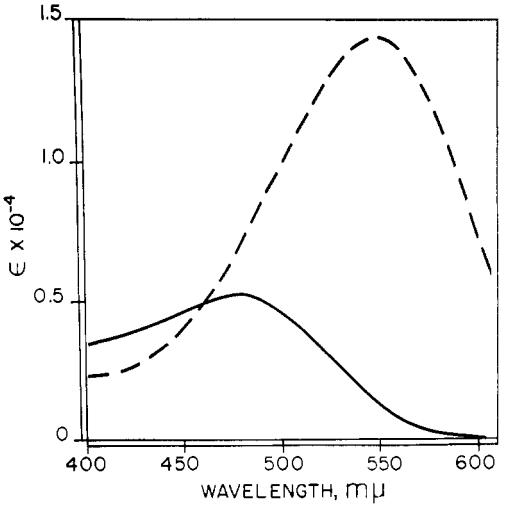

FIGURE 2: Absorption spectra of monoazo-*N*-acetyltyrosine (——) and bisazo-*N*-acetyltyrosine (---), bicarbonate buffer, pH 8.8. The derivatives were obtained by treating 1×10^{-4} M *N*-acetyltyrosine with 1.2×10^{-4} M DHT and with 3×10^{-2} M DHT, respectively, 30 min, room temperature.

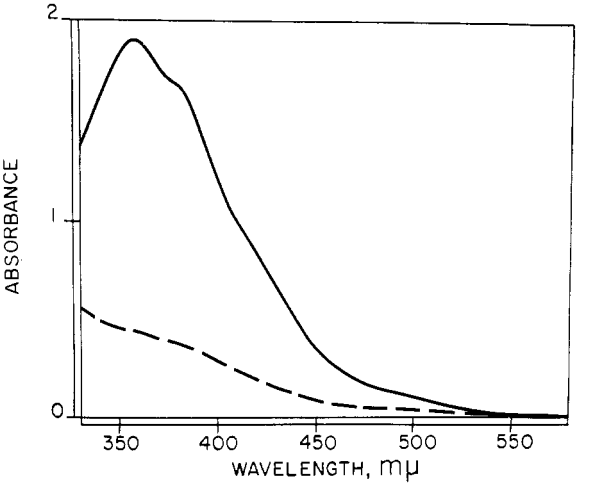

FIGURE 3: Absorption spectra on reaction of 1.6×10^{-4} M histidine (---) and *N*-acetylhistidine (——) with 2×10^{-4} M DHT, bicarbonate buffer, pH 8.8, 30 min, room temperature.

to yield the monoazotyrosine derivative. The concentration of the monoazotyrosyl derivative was estimated from the absorbance at 478 mμ, based on a molar absorptivity of 5.1×10^3.

Coupling Reaction. A solution of DHT (1–3 ml) at the desired concentration was mixed with a solution of amino acid derivative or peptide ($1–3 \times 10^{-5}$) in 1 M $KHCO_3$, pH 8.8, diluted to 10 ml with 1 M $KHCO_3$, and the pH was immediately readjusted to 8.8 if necessary. The reaction mixtures were kept at room temperature for 30 min before spectra were recorded.

Results

At pH 8.8, and dependent upon the molar ratios of reactants, the coupling of DHT with histidyl and tyrosyl residues results in absorption bands characteristic of the mono- and bisazo derivatives of *N*-acetylhistidine with maxima at 360 and 480 mμ (Figure 1) and of the corresponding *N*-acetyltyrosine with maxima at 478 and 548 mμ (Figure 2), respectively.

The product of the reaction of DHT with *N*-acetylhistidine at a molar ratio of 1:1 has a spectrum with a maximum at 359–360 mμ and a shoulder with a midpoint at 375 mμ, while the corresponding monoazo-*N*-acetyltyrosine derivative absorbs maximally at 478 mμ. Both of these spectra are virtually identical with those obtained on reaction of DHT with a large molar excess of either *N*-acetylhistidine or *N*-acetyltyrosine, conditions known to lead solely to the formation of the monoazo derivatives. The molar absorptivity calculated from the concentration of monoazotyrosine formed under these conditions is in close agreement with that observed with authentic monoazo-*N*-carbobenzoxytyrosine (Table I). The addition of a higher molar excess of DHT converts *N*-acetyltyrosine or *N*-acetylhistidine to the bisazo derivatives accompanied by shifts in the absorption maxima to 480 mμ for bisazohistidine (Figure 1) and to 548 mμ for bisazotyrosine (Figure 2).

TABLE I: Azo Derivatives of Histidine, Tyrosine, Histidyl, and Tyrosyl Residues: Wavelengths of Maximal Absorption (λ_{max}) and Molar Absorptivities (ϵ).[a]

	Monoazo		Bisazo	
	λ_{max} (mμ)	$\epsilon \times 10^{-3}$	λ_{max} (mμ)	$\epsilon \times 10^{-4}$
Histidine	360	2.9	480	1.95
N-Acetylhistidine	360	11.5	480	2.10
Tyrosine	478	2.9	548	0.13
N-Acetyltyrosine	478	5.2	548	1.41
N-Carbobenzoxy-tyrosine	478	5.1	548	1.42
N-Benzoyltyrosine	478	5.1	548	1.42
N-Acetyltyrosine ethyl ester	478	5.2	548	1.39
N-Acetyltyrosine amide	478	5.2	548	1.40
N-Glycyltyrosine amide	478	5.2	548	1.41

[a] Coupling with DHT was carried out at room temperature, pH 8.8. For the monoazo derivatives the molar ratio of the reactants was 1:1. For the bisazo derivatives a 300-fold molar excess of DHT was added. The spectra of the reaction mixture were recorded after 30 min.

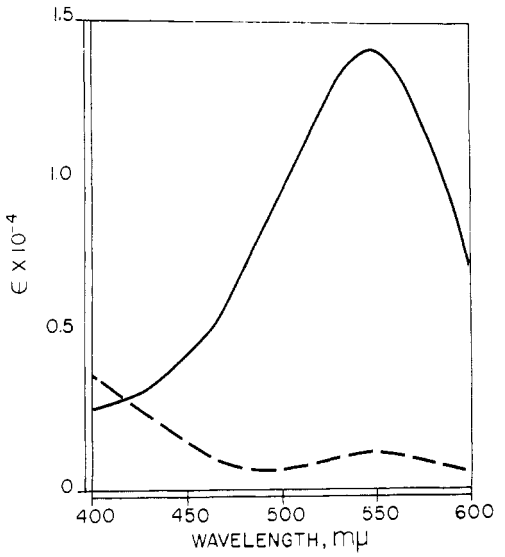

FIGURE 4: Absorption spectra of bisazo-*N*-acetyltyrosine (——) and bisazotyrosine (---), bicarbonate buffer, pH 8.8, 1 × 10^{-4} M. *N*-Acetyltyrosine and tyrosine were treated with a 300-fold molar excess of DHT, 30 min, room temperature.

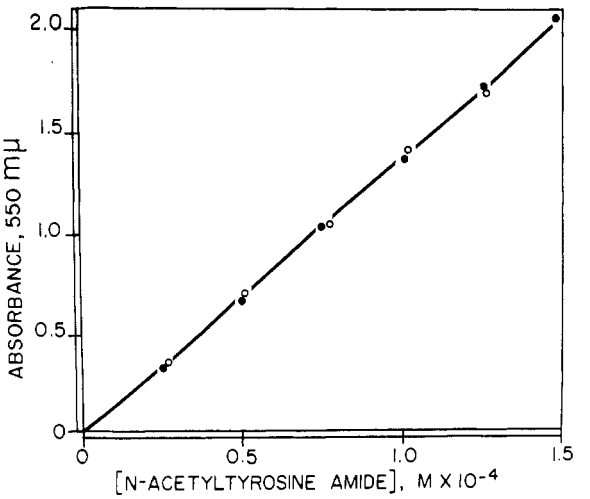

FIGURE 5: The relation between color development at 550 mμ and concentration of *N*-acetyltyrosine amide, following reaction with a 300-fold molar excess of DHT (●) in bicarbonate buffer, pH 8.8, 30 min, room temperature. The open circles (○) represent experiments carried out in the presence of various amino acid derivatives and peptides (see the text). Analogous results are obtained with *N*-acetylhistidine and color development is measured at 480 mμ.

The absorption maxima of azohistidine and azotyrosine are identical with those of the corresponding derivatives of the *N*-acetylamino acids, but the molar absorptivities of the monoazohistidyl and bisazotyrosyl derivatives differ markedly (Figures 3 and 4). Thus, when identical concentrations of histidine and *N*-acetylhistidine are treated with the same amount of DHT at 8.8, there is no shift in the absorption maximum, but the absorbance of monoazo-*N*-acetylhistidine is greater (ϵ 1.15 × 10^4) than that of monoazohistidine (ϵ 2.9 × 10^3). The differences are even more striking when a 200–300-fold molar excess of diazo reagent is treated with the same concentrations of tyrosine and *N*-acetyltyrosine (Figure 4). The molar absorptivity of bisazo-*N*-acetyltyrosine at 548 mμ is 1.41 × 10^4 while that of bisazotyrosine is 1.3 × 10^3. Esterification or amidation of the carboxyl group does not affect the absorption (Table I). The bisazo derivative of *p*-hydroxy-β-phenylpropionic acid absorbs maximally at 549 mμ with a molar absorptivity of 1.45 × 10^4, almost the same as that of bisazo-*N*-acetyltyrosine.

When treated with DHT, histidine and tyrosine and their analogs are converted into their bisazo derivatives at different rates. Thus, within a few minutes, the addition of a 150-fold excess of reagent to *N*-acetylhistidine quantitatively yields the bisazo derivative. *N*-Acetyltyrosine amide, however, under these conditions yields a mixture of mono- and bisazo derivatives, with absorption maxima intermediate between the monoazo (478 mμ) and bisazo (548 mμ) derivatives (Table II). Thus, substitution of one azo group in the aromatic nucleus of tyrosine retards subsequent substitution. Even with a diazo reagent:tyrosine ratio as high as 300, complete formation of bisazotyrosine requires about 15 min; the corresponding *N*-acetylhistidine derivative is formed in 1 min.

TABLE II: The Wavelengths of Maximal Absorption as a Function of Addition of Increasing Amounts of DHT to *N*-Acetylhistidine or *N*-Acetyltyrosine.

Excess of DHT to Reactant (moles/mole)	*N*-Acetylhistidine (mμ)	*N*-Acetyltyrosine (mμ)
1	360	478
3	360	478
5	360	478
10	360, 480	478
50	480	500
150	480	535
300	480	548

The mono- and bisazo derivatives of histidine and tyrosine are stable for several days at room temperature, pH 8.8. They are destroyed under standard conditions for protein hydrolysis, *i.e.*, in 24 hr 6 N HCl at 105°, and the free amino acids are not regenerated. Thus, the number of histidyl and tyrosyl residues modified in a protein or peptide can be determined by measuring their loss on acid hydrolysis as compared to the native protein.

On reaction of *N*,*O*-diacetyltyrosine with DHT,

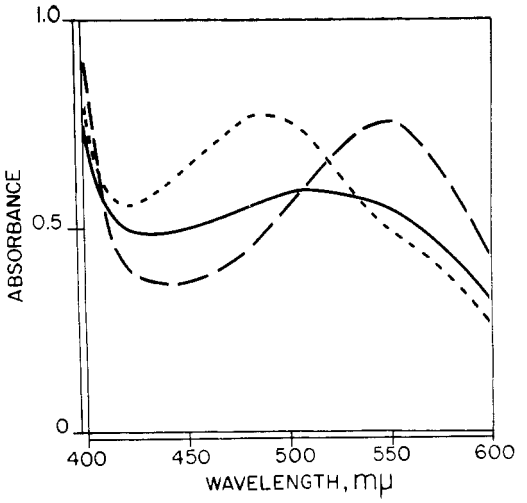

FIGURE 6: Absorption spectra of some azoproteins in bicarbonate buffer, pH 8.8. Pepsin (– –) 3.33 × 10^{-6} M, ribonuclease (----) 5.8 × 10^{-6} M, and trypsin (——) 4.25 × 10^{-6} M were treated with a large excess of DHT. Condition as described in Method A.

less than 5% of the bisazo compound forms. This observation supports the suggestion that the presence of a free phenolic group is necessary for the coupling reaction to occur.

At pH 8.8, a 300-fold or greater molar excess of DHT quantitatively converts *N*-acetylhistidine and *N*-acetyltyrosine to the bisazo derivatives which maximally absorb at 480 and 548 mμ, respectively. The product of the coupling reaction, determined at the appropriate wavelength, with these acylamino acids is directly proportional to their concentrations over a range of from 1 × 10^{-5} to 1.5 × 10^{-4} M (Figure 5). Neither the quantitative yield nor the molar absorptivities are affected when a mixture containing the peptides hippuryllysine, hippurylarginine, hippurylphenylalanine, glycylleucine, *N*-carbobenzoxyglycyltryptophan, and glutathione and the acylamino acids *N*-acetylserine and *N*-acetylthreonine is added to *N*-acetylhistidine or *N*-acetyltyrosine (each of these at twice the concentration of the latter) prior to the addition of the diazo reagent (Figure 5). Since the DHT derivatives of other amino acid residues do not exhibit absorption at these wavelengths, those of both histidine and tyrosine can be determined in such mixtures, as long as a sufficient excess of the reagent is added.

The absorption of bisazohistidine at 548 mμ is very low; hence, it does not markedly affect the quantitative determination of bisazotyrosine at this wavelength. In contrast, bisazotyrosine absorbs significantly at 480 mμ, the wavelength of maximal absorption of bisazohistidine resulting in interference with the quantitative determination of the latter. However, the absorbance of bisazotyrosine at 480 mμ is just about one-half of that at its maximum, *i.e.*, at 550 mμ (Figure 2). Hence, measurement of the absorbance at 550 mμ establishes the amount of bisazotyrosine formed. This, in turn, permits the determination of its contribution to absorbance at 480 mμ. Subtraction of this value from the total absorbance at 480 mμ should then give the absorbance due to bisazohistidine and, hence, the concentrations of the individual components.

Mixtures of the *N*-acetylhistidine and *N*-acetyltyrosine were treated with a large excess of DHT for 30 min, pH 8.8, at room temperature. To test the analytical validity, the absorption was measured at 550 and 480 mμ. When molar ratios of histidine to tyrosine were varied from 1:6 to 6:1, the error in the quantitative determination of histidine and tyrosine never exceeded 5%.[3]

The possibility that reaction of DHT with tyrosyl and histidyl residues of peptides would yield different values either of λ_{max}, of ϵ, or of both was tested next with synthetic peptides. The addition of a low concentration of DHT to the octapeptide Asn-Arg-Val-Tyr-Val-His-Pro-Phe at pH 8.8 resulted in the appearance of absorption maxima at 360 and 480 mμ, characteristic of monoazohistidine and monoazotyrosine, respectively. Increase in the concentration of DHT generated the bisazohistidyl and -tyrosyl peptide with an absorption maximum at 480 mμ (ϵ 2.76 × 10^4) and a shoulder at 550 mμ (ϵ 1.48 × 10^4). Further, the histidyl and tyrosyl residues of the peptide Asn-Arg-Val-Phe-Val-His-Pro-Phe and of β^{1-24}-corticotropin are completely converted to their bisazo derivatives. For all of these the molar absorptivity of the bisazohistidyl residues is 2.05 × 10^4 at 480 mμ and that of the bisazotyrosyl residues 1.38 × 10^4 at 550 mμ. The data demonstrate the simultaneous and quantitative measurement of the histidine and tyrosine content of these peptides since these molar absorptivities correspond exactly to those found for the model compounds (Table III).

Similar studies were performed on several proteins whose histidine and tyrosine contents are known (Figure 6 and Table IV). In all instances, the proteins, 6 × 10^{-6}–1 × 10^{-5} M, were treated for 90 min with an approximately 3000-fold molar excess of DHT, pH 8.8. The tyrosyl and histidyl residues of insulin and chymotrypsin were modified quantitatively, but a smaller number than that known to be present from amino acid analyses was altered in trypsin, ribonuclease, and pepsin. Thus, all histidyl and tyrosyl residues are not equally reactive to DHT. If the three-dimensional structures of these proteins preclude complete reaction, then denaturation, followed by coupling, might lead to quantitative modification. Under these circumstances, all of the histidine and tyrosine residues of trypsin, ribonuclease, and pepsin were indeed found to react. Since both urea and guanidine react with DHT, alkali was employed to denature proteins. In at least one instance, ovalbumin, even alkali treatment did

[3] Since the absorption of bisazotyrosine at 600 mμ is 1.06 of that at 480 mμ, this factor can be useful in double checking the absorbance to be subtracted from that at 480 mμ. This is especially important in proteins with a high histidyl content, which will contribute significantly to the absorbance at 550 mμ.

TABLE III: Molar Absorptivities[a] of Bisazohistidyl and -tyrosyl Derivatives.

	ε at 480 mμ	ε/His	ε at 550 mμ	ε/Tyr
(1) *N*-Acetyl-His	2.11	2.11	0.11	...
(2) *N*-Acetyl-Tyr	0.70	...	1.40	1.40
(3) Asp-Arg-Val-Phe-Val-His-Pro-Phe	2.05	2.05	1.25	...
(4) Asp-Arg-Val-Tyr-Val-His-Pro-Phe	2.76	2.06	1.47	1.37
(5) Val-Tyr-Val-His-Pro-Phe	2.76	2.06	1.48	1.38
(6) β^{1-24}-Corticotropin	3.43	2.03	2.88	1.38

[a] The molar absorptivities ($\times 10^{-4}$) of 4–6 were calculated as described in the text. β^{1-24}-Corticotropin contains one histidyl and two tyrosyl residues.

TABLE IV: Numbers of Tyrosyl and Histidyl Residues in Various Proteins as Determined by Coupling with DHT.[a]

	Native	Alkali Denatured	Lit.
Tyrosine			
Insulin	3.6	3.9	4[b]
Chymotrypsin	3.8	3.9	4[c]
Trypsin	7.8	9.5	10[d]
Ovalbumin	1.8	9.6[e]	10[f]
Ribonuclease	3.3	6.0	6[h]
Pepsin	14.2	16.0	16–17[g]
Histidine			
Insulin	1.7	1.8	2[b]
Chymotrypsin	2.0	2.1	2[c]
Trypsin	2.9	2.9	3[d]
Ovalbumin	2.1	5.8[e]	6[h]
Ribonuclease	2.5	4.1	4[h]
Pepsin	1.1	1.2	1[g]

[a] Determined by Method A; see text for details. [b] Ryle *et al.* (1955). [c] Wilcox *et al.* (1957). [d] Walsh *et al.* (1964). [e] Determined by Method B. [f] Tristram and Smith (1963). [g] Blumenfeld and Perlmann (1958). [h] Smyth *et al.* (1963).

not result in complete reaction of all the known tyrosines and histidines with DHT. This could be achieved, however, if the alkali treatment was followed by acetylation with acetic anhydride (Table IV). These findings form the basis for two methods, A and B, for the determination of the total histidine and tyrosine content of proteins using DHT, as follows.

Determination of Histidine and Tyrosine in Proteins.

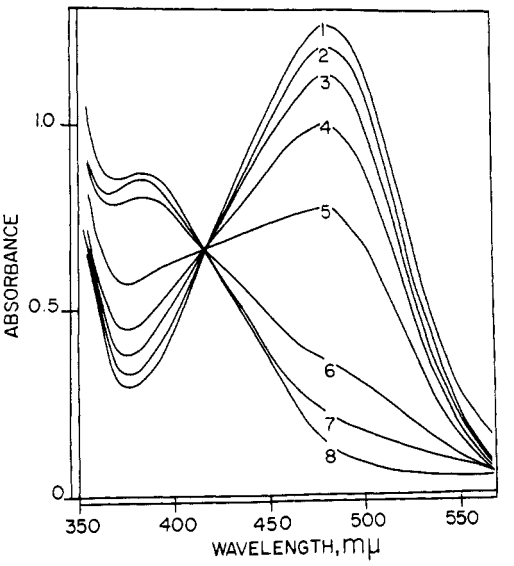

FIGURE 7: Effect of pH on the absorption spectrum of monoazo-*N*-carbobenzoxytyrosine. (1) 0.01 N NaOH; (2) pH 10.5; (3) pH 10.0; (4) pH 9.5; (5) pH 8.8; (6) pH 8.5; (7) pH 8.2; (8) pH 6.4. Above pH 8.0, buffers were prepared using 0.5 M potassium bicarbonate. Below pH 8.0, 0.1 M sodium phosphate was used.

METHOD A.[3] An aliquot of protein (0.1–0.2 ml) is diluted to 1 ml with 0.5 M NaOH. The reaction mixture is kept at room temperature for 30 min. The sample is then diluted with 7 ml of 1 M $KHCO_3$, pH 8.8, and 2 ml of DHT, 0.16–0.2 M. The final protein concentration should be 6×10^{-6}–1×10^{-5} M. After 10 min at room temperature another 2-ml aliquot of DHT is added, and the reaction mixture is allowed to stand for 90 min. If necessary, the pH is readjusted to 8.8 after each addition. The absorbance at 480 and 550 mμ is measured against a buffer blank. The tyrosine content is calculated from the absorbance at 550 mμ and its molar concentration is given by $A_{550}/1.38 \times 10^4$. The histidine content is determined from the absorbance at 480 mμ subtracting the contribution of tyrosine at that wavelength, which is one-half that at 550 mμ. The molar concentration of histidine is therefore $(A_{480} - A_{550/2})/(2.05 \times 10^4)$.

METHOD B.[3] Protein (0.3–0.5 μM) is incubated in 1 ml of 0.5 M NaOH for 30 min and then diluted to 3 ml with 1×10^{-3} M Tris, pH 7.5. The solution is cooled to 0° and 35 μl of acetic anhydride added. The pH is maintained at 7.5 by means of a pH-Stat. After 20 min another 25 μl of acetic anhydride is added. After 15 min, the pH is raised to 13. The reaction mixture is kept at room temperature for 20–30 min to allow deacetylation of *O*-acetyltyrosine. The protein solution is then adjusted to a final volume of 5 ml. A 1-ml aliquot is diluted with 7 ml of 1 M $KHCO_3$, pH 8.8, and 2 ml of DHT, 0.16–0.2 M. The final pH should be 8.8 to 9.0. After 20 min at room temperature another 2 ml of the DHT solution is added and the reaction

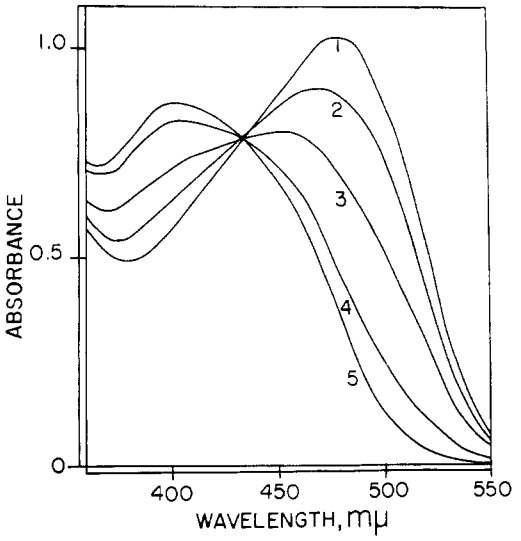

FIGURE 8: Effect of pH on the absorption spectrum of bisazo-*N*-acetylhistidine. (1) pH 8.8–11; (2) pH 8.0; (3) pH 7.6; (4) pH 7.0; (5) pH 6.4. Above 8.0, buffers were prepared using 0.5 M potassium bicarbonate. Below pH 8.0, 0.1 M sodium phosphate was used.

mixture is allowed to stand at room temperature for 90 min. The absorbance at 480 and 550 mμ is measured against a buffer blank. The tyrosine and histidine content is calculated as above.

It is important to maintain the pH at 8.8 since both λ_{max} and molar absorptivity change as a function of pH. The effect of pH on the absorption spectrum of monoazo-*N*-carbobenzoxytyrosine is shown in Figure 7. The maximum of the fully ionized form, at 478 mμ, shifts to 385 mμ on lowering the pH below 8.8; the isosbestic point is at 416 mμ. Similarly, at pH's below 8.8, the absorption maximum of bisazo-*N*-acetyltyrosine amide decreases and the absorption at shorter wavelengths correspondingly increases. The absorption maximum of bisazo-*N*-acetylhistidine shifts from 480 (at pH 8.8) to 400 mμ (at pH values below 8). In this instance, the isosbestic point is at 432 mμ (Figure 8).

Discussion

Diazonium compounds induce visible color on reaction with proteins, thus providing a major analytical asset. However, incomplete resolution of the spectral bands of the various azo derivatives of amino acids has limited the usefulness of these reagents. Recently, Horinishi *et al.* (1964) introduced diazonium-1H-tetrazole as a new coupling reagent for proteins. The absorption bands of monoazotyrosine, bisazotyrosine, and bisazohistidine were reported to be resolved, with maxima at 380, 550, and 480 mμ, respectively, apparently obviating the spectral shortcomings of similar reagents employed in the past. Histidine was converted rapidly to the bisazo derivative. Hence, this compound was suggested for the determination both of the number of "reactive" histidyl residues in native proteins and of those which react only after denaturation. Histidine and tyrosine, the free amino acids, were employed as model compounds for the reaction with DHT to interpret the spectra obtained for azoproteins. The present studies indicate that the molar absorptivities of the azo derivatives of N-substituted tyrosine and histidine differ from those of the *free* amino acids; the interpretation of spectra obtained on proteins modified with DHT must be considered in the light of this observation.

The concentration of DHT has been measured based on its reaction with excess histidine (Horinishi *et al.*, 1964). Under the conditions suggested we consistently find a value of ϵ 2.91×10^3, not 1.2×10^4 as reported. This discrepancy remains unexplained. However, monoazo-*N*-acetylhistidine prepared under analogous conditions absorbs maximally at λ 360 mμ and ϵ 1.15×10^4. The different molar absorptivities for histidine and *N*-acetylhistidine which we have obtained might be anticipated, since interactions of diazonium reagents with free α-amino groups are known (Howard and Wild, 1957; Tabachnick and Sobotka, 1959, 1960) and would be expected to alter the stoichiometry.

Similar considerations apply to the use of free tyrosine as a standard. Reaction of a 300-fold molar excess of the diazonium reagent with tyrosyl derivatives, *e.g.*, *N*-acetyltyrosine and *N*-acetyltyrosine amide, results in a colored derivative, presumably the bisazo form, which absorbs maximally at 548 mμ, similar to that of free tyrosine already reported; however, the molar absorptivity, ϵ 1.41×10^4, is much higher than that of the derivative of free tyrosine, ϵ 1.3×10^3 (Table III). The spectrum of the azo derivative of *p*-hydroxy-β-phenylpropionic acid is identical with that of either *N*-acetyltyrosine or *N*-acetyltyrosine amide. Since this compound lacks only the α-amino group of tyrosine, the data suggest that the absorptivity difference is due to this group. The α-amino and carboxyl groups of virtually all of the tyrosyl residues of protein are blocked; hence, their bisazo derivatives should exhibit spectra similar to that of bisazo-*N*-acetyltyrosine (amide) (Table I).

Monoazo-*N*-carbobenzoxytyrosine was synthesized to serve as a suitable spectrophotometric standard. This derivative absorbs maximally at 478 mμ (Table I) where DHT does not absorb, obviating spectral interference from the reagent. The spectral properties of this synthetic product were identical with those of other *N*-acyltyrosyl derivatives (Table I) prepared by coupling with an amount of DHT equimolar or slightly greater than equimolar. In all instances (Table I), maximal absorption was found at 478 mμ, not at 380 mμ as reported (Horinishi *et al.*, 1964). The molar absorptivity, ϵ, was 5.1×10^3.

Tyrosine can be determined directly by measuring the absorbance at 548 mμ, since bisazohistidine absorbs maximally at 480 mμ and only insignificantly at 548 mμ. Measurements at both wavelengths permit the determination of both tyrosine and histidine if condi-

tions are employed which ensure complete formation of the bisazo derivatives.[4]

Histidine concentration can only be determined at 480 mμ if the contribution of tyrosine to the absorption at this wavelength is known, since both bisazotyrosine and bisazohistidine absorb significantly here. This contribution can be determined very conveniently, however, since the absorption of bisazotyrosine at 480 mμ is one-half that at 550 mμ; hence, it proved advantageous to measure bisazotyrosine at 550 rather than at 548 mμ. On this basis, the contribution of the absorption at 480 mμ, due to tyrosine, can be calculated and subtracted.[3] Since monoazotyrosine also absorbs at 480 mμ, this procedure is legitimate only when both tyrosine and histidine are known to have been converted quantitatively to the bisazo derivatives.

The concentration of the reagent is an important feature of the reaction as here employed. DHT must be used in large molar excess to ensure complete formation of the bisazo derivatives. Like other diazonium compounds (Howard and Wild, 1957; Higgins and Harrington, 1959; Tabachnick and Sobotka, 1960) DHT decomposes—albeit slowly—and, hence, the concentration of the reagent may change on standing. Further, it may react with functional groups of other amino acids, *e.g.*, lysine, arginine, serine, and threonine. The coupled products of these amino acids are colorless and, therefore, do not interfere spectrally; however, a large fraction of the reagent may be consumed in these reactions. When an adequate excess of DHT is used, the histidyl and tyrosyl residues of peptides such as Asn-Arg-Val-Phe-Val-His-Pro-Phe, Asn-Arg-Val-Tyr-Val-His-Pro-Phe, and β^{1-24}-corticotropin are converted completely to the bisazo derivatives (Table III).

To be effective in bringing about quantitative coupling, all of the histidyl and tyrosyl residues of proteins must be accessible to the reagent. The potential suitability of this procedure for the determination of free histidyl residues of proteins (Horinishi *et al.*, 1964) is rendered ambiguous largely by the spectral considerations already detailed (*vide supra*). DHT is suitable, however, for the determination of the total number of histidines and tyrosines present, if these are rendered accessible to the reagent by prior denaturation, as shown by the results obtained with several proteins (Table IV). In general, denaturation with alkali allows reaction of all of the residues. In at least one protein, *i.e.*, ovalbumin, however, even this did not lead to complete coupling. In this instance, acetylation of the amino groups subsequent to denaturation allowed the reaction to go to completion.

The major spectral difficulty in employing DHT as a reagent for histidine arises from its simultaneous interaction with tyrosine. This can be eliminated by prior *O*-acetylation of these residues which precludes their coupling with DHT. This procedure has been employed successfully in this laboratory (Sokolovsky and Vallee, 1966). Amino acid analysis will serve, of course, as a more definitive index both of the identity and number of histidyl, tyrosyl, and other residues modified. The combination of these and other approaches has led to extensions of the use of this reagent (M. Sokolovsky and B. L. Vallee, in preparation).

[4] The absorbance at 480 mμ (the "plateau level"), thought to reflect exclusively the numbers of histidyl residues of proteins (Horinishi *et al.*, 1964), appears to incorporate contributions of both mono- and bisazotyrosyl residues. Increments of DHT resulted in slight increases of absorbance at 480 mμ, and marked increases at 550 mμ. This would be observed if the underlying reaction is monoazo → bisazotyrosine.

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