similar to those reported here. Consequently, it may prove possible to use these general spectral properties to characterize proteins azo coupled with a large variety of structurally different diazonium salts, providing pure and well-characterized model compounds are available. The utilization of the spectra to characterize azo proteins is presented in the following paper.

Registry No. NAMAAT, 39927-13-4; NABAAT, 88229-06-5; NAMAAH(C-2)·$^3/_2$Ba, 88211-97-6; NAMAAH(C-4)·$^3/_2$Ca, 88211-98-7; NABAAH·2Ca, 88229-07-6; NAMSAT·Ba, 88212-00-4; NABSAT·Ba, 88212-01-5; NAMSAH(C-2)·Ba, 88212-02-6; NAMSAH(C-4)·xBa, 88212-03-7; NABSAH, 88212-04-8; arsanilic acid, 98-50-0; arsanilic acid diazonium bromide, 88211-96-5; *N*-acetyl-L-tyrosine, 537-55-3; N^{α}-acetyl-L-histidine, 2497-02-1; sulfanilic acid, 121-57-3; sulfanilic acid diazonium bromide, 88211-99-8.

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Preparation and Characterization of Sulfanilazo and Arsanilazo Proteins†

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ABSTRACT: A thorough study of the susceptibility of a series of proteins to azo coupling as a function of pH, ratio of diazonium salt to protein, and type of diazonium salt was undertaken. Spectral analysis of the azo proteins indicates that tyrosine is modified in preference to histidine, that while both possible structural isomers of azohistidine can be formed, azo coupling at the C-2 position is more common, and that little if any bis coupling occurs. Spectral analysis is based on data from chromatographically pure and chemically analyzed azo amino acids. Use of diazotized [^{35}S]sulfanilic acid indicates that the majority of residues modified in azo proteins is not detected by spectral analysis. These residues were identified as the products of the reaction of diazonium salt with free amines (i.e., N-terminal and/or the ϵ-amine of lysines) and with sulfhydryl groups. The former reactions are decreased or eliminated by acylation prior to azo coupling of the protein. The extent of azo coupling was found to be characteristic of the particular protein and not simply a function of the total number of potentially reactive residues in a protein.

Eighty years ago, Pauly (1904) reported the use of aromatic diazonium salts for the modification of proteins. Diazonium salts are known to react with various amino acid side chains of proteins to produce chromophores that absorb between 300 and 600 nm. Azo coupling of proteins[1] has found application in immunological investigations (Landsteiner & Lampl, 1917; Thorpe & Singer, 1969), the determination of structure–function relationships (Suh & Kaiser, 1976; Alter & Vallee, 1978; Fairclough & Vallee, 1970; Gorecki et al., 1978; Cueni & Riordan, 1978), the study of membrane proteins (Bell et al., 1979), a method for immobilizing proteins transferred to paper after electrophoresis (Renart et al., 1979), tumor imaging (Sundberg et al., 1974), and a novel approach to cancer therapy (Mizusawa et al., 1982). In a number of instances, it appears that azo coupling is specific for particular hyperreactive protein residues. We are interested in this modification as a particularly promising method for producing chelating agents on proteins. Once formed, substitution-inert Co(III) can be specifically incorporated into these chelating sites (Urdea & Legg, 1979a,b; Urdea et al., 1979).

Ascertaining conditions necessary for specific coupling of aryl diazonium salts to proteins requires a simple, accurate method for determining the type and number of residues modified. The method of choice is visible spectral analysis. However, attempts to utilize the spectral characteristics of simple azo model compounds to determine the extent of tyrosine and histidine modification in azo proteins (Tabachnick

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[1] The terms "diazotization" and "diazotized" have been used in previous work to indicate both the formation and coupling of diazonium salts. However, in this paper, in accordance with current usage, these terms only refer to diazonium salts while "azo coupling" and "coupled" refer to the reaction of diazonium salts.

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& Sobotka, 1959, 1960)[2] have been, until now, subject to considerable error since the model compounds were incorrectly characterized (Pielak et al., 1984). In the preceding paper we reported the spectral properties of pure mono- and bis(arsanilazo) and mono- and bis(sulfanilazo) derivatives of tyrosine and histidine. Here, we report the application of these properties to the determination of the extent and specificity of histidine and tyrosine azo coupling in proteins. The concomitant modification of lysine and cysteine is also considered.

Experimental Procedures

Proteins and Chemicals. RIA-grade bovine serum albumin (BSA),[3] bovine erythrocyte carbonic anhydrases A and B, 3 times crystallized bovine pancreatic α-chymotrypsin, types IA and XIIA pancreatic ribonuclease A (RNase), thermolysin, poly(L-lysine) (M_r 20 000), and yeast alcohol dehydrogenase (YADH) were purchased from Sigma and used without further purification. Jack bean concanavalin A (Ca^{2+},Mn^{2+} form) (Con A) was a gift from Dr. J. A. Magnuson of this department. Bovine insulin (Sigma) was recrystallized from 0.01 M HCl. Bovine pancreatic carboxypeptidase A (Sigma) was purified as described by Urdea & Legg (1979a). Trinitrobenzenesulfonic acid (TNBS) was recrystallized as described by Fields (1972). Carrier-free $H_2{}^{35}SO_4$ was obtained from New England Nuclear. All other chemicals were used without further purification.

Protein Concentrations. The following molar absorptivities and $A^{1\%}$ values were used to determine the concentration of the native proteins: carboxypeptidase A, 6.42×10^4 M^{-1} cm^{-1} at 278 nm (Simpson et al., 1963); thermolysin, 6.63×10^4 M^{-1} cm^{-1} at 280 nm (Matsumbara, 1970); BSA, 4.69×10^4 M^{-1} cm^{-1} at 278 nm (Koberstein et al., 1968); α-chymotrypsin, $A^{1\%}_{280}$ = 20.0 (Wilcox et al., 1957); carbonic anhydrases A and B, 5.2×10^4 M^{-1} cm^{-1} at 280 nm (Carpy, 1968); Con A, $A^{1\%}_{280}$ = 13.7 (Yariv et al., 1968); YADH, 2.08×10^5 M^{-1} cm^{-1} at 278 nm (Koberstein et al., 1968); RNase, 1.03×10^4 M^{-1} cm^{-1} at 279 nm (Koberstein et al., 1963); insulin, 5950 M^{-1} cm^{-1} at 276 nm (Hamlin & Arquilla, 1974). All data are expressed in terms of the monomeric protein.

Analytical Procedures. Sulfhydryls were determined with DTNB (Ellman, 1959) and primary amines with TNBS (Fields, 1972). Concentrations of azo proteins were determined by either the method described under Spectral Analysis or the methods of Bradford (1976) or Lowry (1951). UV–visible spectra were recorded on a Cary 219 spectrophotometer (spectral band width of 1.0 nm); pH was controlled and measured with a pH stat designed in this laboratory (Warner et al., 1980) with a Radiometer GK 2321C electrode. ^1H NMR spectra were obtained on a JEOL JNM-MH-100 spectrometer. A Beckman LS230 scintillation counter set on the wide ^{14}C channel was used to quantitate ^{35}S. All samples were counted to a precision of ±1% in 6.5 mL of ACS (Amersham), with an efficiency of greater than 90%.

Synthesis of [^{35}S]Sulfanilic Acid. Commercial [^{35}S]sulfanilic acid (from New England Nuclear and Amersham) was not pure, as established by radiography–TLC in the ISTEA (Warner & Legg, 1979). The synthesis below is an adaptation of a large-scale industrial process (Fierz-David & Blangey, 1949).

A solution of $H_2{}^{35}SO_4$ prepared by adding 165 μL of 96.3% H_2SO_4 (3.0 mmol) to 1 mL of carrier-free $H_2{}^{35}SO_4$ (5 mCi, 3.3 nmol) was *slowly* added to 279 mg (3.0 mmol) of freshly distilled aniline in a 5-mL glass vial and vortexed for 1 min. After lyophilization, the residue was heated in vacuo (less than 4 mmHg) in a sand bath at 200 °C. After 6 h, the vial was cooled, and 2.2 mL of water and 0.2 g of Na_2CO_3 were added. The solution plus a 1-mL wash was filtered (0.1 μm pore size, Millipore Corp.). Crystallization was induced by addition of ca. 4 drops of concentrated HCl. The crystals were collected by centrifugation. This regimen was repeated until the pH of the mother liquor was less than 2. The combined purple/brown crystals (269 mg) were dissolved in 4.5 mL of boiling water and recrystallized with stirring at 4 °C. The fine, white crystals were washed with ethanol and anhydrous ether and dried at 115 °C for 30 min. The yield of pure ^{35}S-labeled sulfanilic acid was 40%, and its specific activity was 3200 cpm/nmol. The product was stored over P_2O_5 at −20 °C.

In order to obtain analytical data, a parallel reaction was performed, substituting 1 mL of water for the carrier-free $H_2{}^{35}SO_4$. Anal. Calcd for $C_6H_7NO_3S$: C, 41.61; N, 8.09; H, 4.08. Found: C, 41.28; N, 8.06; H, 4.11. The compound exhibited one fluorescamine-positive spot (R_f 0.54) in 80/20 ISTEA on Whatman silica gel plates (0.2-mm layer thickness) coinciding with pure unlabeled sulfanilic acid (Eastman). The ^1H NMR spectra of the synthetic and commercial material were indistinguishable. Extraction of the synthetic sample with 0.3 M HCl followed by addition of $BaCl_2$ to the supernatant demonstrated the absence of free sulfate.

The [^{35}S]sulfanilic acid was pure as determined by TLC (radiography and fluorescamine tests). Upon reaction of the diazonium salt of the [^{35}S]sulfanilic acid with a large excess of BSA as described in the following section, 100% of the radioactivity was bound to BSA. Commercial unlabeled and synthetic [^{35}S]sulfanilic acid were also indistinguishable in terms of their reaction with BSA as shown by visible spectral analysis.

Generation of Diazonium Salts and Azo Coupling of Proteins. All solutions were freshly prepared. An aqueous solution of $NaNO_2$ (8.3 mg/mL) was added to an equal volume of a stirred slurry of sulfanilic acid (20.8 mg/mL in 0.3 M HCl) in an ice bath. After 30 min, the diazonium salt began to crystallize. The diazonium salt of arsanilic acid was generated and handled as described above except that 26.0 mg/mL arsanilic acid was used. Both arsanilic acid and its diazonium salt are soluble under these conditions. Since diazonium salts are unstable, they were used within 15 min of generation.

A portion of the 60 mM stock diazonium salt solution was pipeted into a stirred, 2.0-mL solution of 0.10 mM protein in an ice bath. Conditions used in each reaction are given in the text or the footnotes of the tables and the figure captions. After 1 h, the reactions were quenched by addition of enough 0.08 M phenol to produce a final concentration equal to that of the original diazonium salt. The reactions were quenched approximately 10 min before further manipulation. The azo protein samples were purified on Sephadex G-25M PD-10 columns (Pharmacia), equilibrated with either 0.02 M 1,4-piperazinediethanesulfonic acid (PIPES)–0.5 M NaCl, pH 7.0, or 0.02 M Na_2CO_3–0.5 M NaCl, pH 11.0, and eluted with the equilibration buffer.

For the ^{35}S incorporation studies, 100 μL of the azo protein reaction mixture was chromatographed on PD-10 columns

[2] The equations of Tabachnick & Sobotka (1960) later appeared (and were cited) in Riordan & Vallee (1972).

[3] Abbreviations: ISTEA, distilled 2-propanol and triethylamine bicarbonate, pH 9.5; BSA, bovine serum albumin; RNase, ribonuclease A; YADH, yeast alcohol dehydrogenase; Con A, concanavalin A; TNBS, trinitrobenzenesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PIPES, 1,4-piperazinediethanesulfonic acid; $(AcO)_2$, acetic anhydride; DMMA, dimethylmaleic anhydride; ^1H NMR, proton nuclear magnetic resonance.

equilibrated in the pH 11 buffer and, 10 6-drop fractions were collected in scintillation vials. The column fractions and triplicate standards of 100 μL of the reaction mixture were counted. The ratio of the average number of cpm in the standards to the excess of diazotized [^{35}S]sulfanilic acid multiplied by the cpm in the column eluant yields the stoichiometry, which was reproducible to within ±3% for duplicate experiments.

Spectral Analysis. An aliquot (300–600 μL) of the azo protein reaction mixture was applied to a PD-10 column equilibrated in either pH 7 or 11 buffer as described above. After the entire azo protein sample entered the column, 30–50 drops of the appropriate buffer were passed through the column and discarded. A 30-drop sample of the pure azo protein was then collected directly into a 1-cm spectrophotometer cell.

Samples were then analyzed for denaturation by measuring the absorbance at 710 nm where none of the azo chromophores absorb. If the absorbance was greater than 0.003, denatured protein was removed with a 0.1-μm filter. Concentration of [^{35}S]sulfanilazo protein was determined by counting triplicate 300-μL aliquots of the solution after spectral analysis. Quantitative dilution was assumed for undenatured arsanilazo proteins. If denaturation was observed, the sample was filtered and the protein concentration determined as described under Analytical Procedures.

The following procedure was used to determine the number of monoazotyrosines, 2-monoazohistidines, and 4-monoazohistidines in an azo protein. Note that all constants pertain to a path length of 1 cm. (A) The molar absorptivity of the azo protein at 380 and 410 nm was obtained at pH 7 in 0.02 M PIPES–0.5 M NaCl (ϵ^7_{380} and ϵ^7_{410}). (B) The molar absorptivity of the azo protein at 540 nm was obtained at pH 11 in 0.02 M Na_2CO_3–0.5 M NaCl (ϵ^{11}_{540}). (C) The stoichiometry of monoazotyrosine was calculated:

$$\text{monoazotyrosine/protein} = \epsilon^{11}_{540}/K_1$$

where $K_1 = 4.79 \times 10^3$ for sulfanilazo proteins and $K_1 = 4.80 \times 10^3$ for arsanilazo proteins. (D) The contribution of the monoazotyrosine to ϵ^7_{380} and ϵ^7_{410} was calculated:

$$\epsilon^7_{380,\text{MAT}} = (\text{monoazotyrosine/protein})K_2$$

$$\epsilon^7_{410,\text{MAT}} = (\text{monoazotyrosine/protein})K_3$$

where $K_2 = 8.18 \times 10^3$ for sulfanilazo proteins, $K_2 = 1.04 \times 10^4$ for arsanilazo proteins, $K_3 = 6.60 \times 10^3$ for sulfanilazo proteins, and $K_3 = 8.04 \times 10^3$ for arsanilazo proteins. (E) The contribution of monoazotyrosine to ϵ^7_{380} and ϵ^7_{410} was subtracted:

$$\epsilon^{7\text{corr}}_{380} = \epsilon^7_{380} - \epsilon^7_{380,\text{MAT}}$$

$$\epsilon^{7\text{corr}}_{410} = \epsilon^7_{410} - \epsilon^7_{410,\text{MAT}}$$

(F) The stoichiometry of 4-monoazohistidine was determined:

$$\text{4-monoazohistidine/protein} = (\epsilon^{7\text{corr}}_{380} - K_4\epsilon^{7\text{corr}}_{410})/K_5 = \text{C-4}$$

where $K_4 = 1.44$ for sulfanilazo proteins, $K_4 = 1.41$ for arsanilazo proteins, $K_5 = 6.02 \times 10^3$ for sulfanilazo proteins, and $K_5 = 9.63 \times 10^3$ for arsanilazo proteins. (G) The stoichiometry of 2-monoazohistidine was determined:

$$\text{2-monoazohistidine/protein} = [\epsilon^{\text{corr}}_{410} - K_6(\text{C-4})]/K_7$$

where $K_6 = 2.90 \times 10^3$ for sulfanilazo proteins, $K_6 = 4.01 \times 10^3$ for arsanilazo proteins, $K_7 = 1.66 \times 10^4$ for sulfanilazo proteins, and $K_7 = 1.84 \times 10^4$ for arsanilazo proteins. Note that if absorbancies are used in the above equations, concentrations of the derivatives are calculated.

Protein Acylation. Protein (0.22–0.30 mM) in 1.3–1.5 mL of 0.02 M PIPES, pH 7.0, was diluted with an equal volume of either saturated sodium acetate for the acetic anhydride [$(AcO)_2$] reactions (Fraenkel-Conrat, 1957) or 0.1 M borate, pH 9.0, for the dimethylmaleic anhydride (DMMA) reactions. Acetic anhydride was added in three or four aliquots at room temperature over 1 h. The pH dropped from 6.6 to 6.0. DMMA reactions (Dixon & Perham, 1968) were carried out in an ice bath with the pH maintained at 9.0 with a pH stat charged with 6 N NaOH. After 90 min, the acylated proteins were repurified, and the buffer was changed to 0.02 M borate–0.5 M NaCl on a PD-10 column. The acylated proteins were then diluted to 0.10 mM and azo coupled immediately.

Results and Discussion

Spectral Analysis. Unlike the previously published method for determining the extent of tyrosine and histidine modification in azo proteins (Tabachnick & Sobotka, 1960), the method developed here is based on the spectral analysis of pure azo amino acids (Pielak et al., 1984). Chromatographic methods developed since the earlier study have been critical to establishing the purity of the reference azo models. In contrast to the earlier studies, the method takes into account both the possible structural isomers of azohistidine. Since the monoazohistidine derivatives do not absorb at 540 nm at pH 11 where monoazotyrosine strongly absorbs (Pielak et al., 1984), the concentration of monoazotyrosine can be independently calculated (see step C of Spectral Analysis under Experimental Procedures). The spectral contribution of the monoazotyrosine at pH 7 in the near-UV, where both monoazohistidines strongly absorb, is then calculated (step D) and subtracted (step E). The concentration of the C-2 and C-4 derivatives is then determined by using their relative absorptions at two wavelengths by solving simultaneous equations (steps F and G). If one assumes that no bisazo derivatives are formed (vide infra), the equations were used to calculate the number of monoazotyrosines and monoazohistidines present in various azo proteins as summarized in Tables I–III.

For these equations to be accurate for azo protein analysis, it must be shown that the azo amino acid models do not associate in solution (Suzuki et al., 1978). Beer's law plots for the individual azo amino acids are linear from at least 1×10^{-6} to 6×10^{-5} M. However, as expected (Harned & Owen, 1958), the visible spectra of the models varied with ionic strength. The pK_a values of the models were lowered by about 0.5 unit in going from water to 0.5 M NaCl. Therefore, it is essential that the equations presented here are used only at the pH values and ionic strengths indicated.

To test the validity of the method, the spectra of the azo proteins were simulated by combining the calculated concentrations of the individual azo models under conditions identical with those used to obtain the spectra of the azo proteins (Figure 1). There is excellent agreement from 320 to 600 nm, considering that the simulations are constrained to fit at only two wavelengths (380 and 410 nm) at pH 7 and one (540 nm) at pH 11. All the simulations obtained for the other azo proteins in the tables fit at least as well as the three examples given in Figure 1.

The relatively poor fit for DMMBSA at pH 11 (Figure 1, center) requires explanation. The azo protein contains comparable amounts of mono(sulfanilazo)tyrosine and 2-mono-(sulfanilazo)histidine (Table II). The pK_a of the imidazole/imidazolate equilibrium for the C-2 derivative in 0.5 M NaCl is 10.5. Therefore, a small microenvironmental effect causing a slight change of this pK_a would have a large effect on the pH 11 spectrum of the azo protein. Evidence for the

Table I: Spectral Analysis and ^{35}S Incorporation Studies of Various Sulfanilazo Proteins

protein[a]	stoichiometry of mono(sulfanilazo) amino acids from spectral analysis				stoichiometry of azo amino acids (^{35}S)	stoichiometry of azo amino acids not detected by spectral analysis
	azotyrosines	azohistidines C-2	azohistidines C-4	total		
BSA	0.62	0.54	0.24	1.40	4.12	2.72
insulin	1.53	0.40	0.00	1.93	2.95	1.02
Con A	1.72	0.51	0.73	2.96	4.29	1.33
RNase	1.27	0.13	0.61	2.01	3.89	1.88
α-chymotrypsin	0.47	0.00	0.04	0.51	3.10	2.59
carbonic anhydrase A	0.47	0.60	0.37	1.44	3.78	2.43
carbonic anhydrase B	0.58	0.69	0.40	1.67	3.51	1.84
thermolysin	2.00	0.03	0.00	2.03	3.07	1.04
carboxypeptidase A	1.01	0.00	0.11	1.12	2.81	1.69

[a] All proteins were coupled with a 5-fold excess of diazotized [^{35}S]sulfanilic acid. See Experimental Procedures for details.

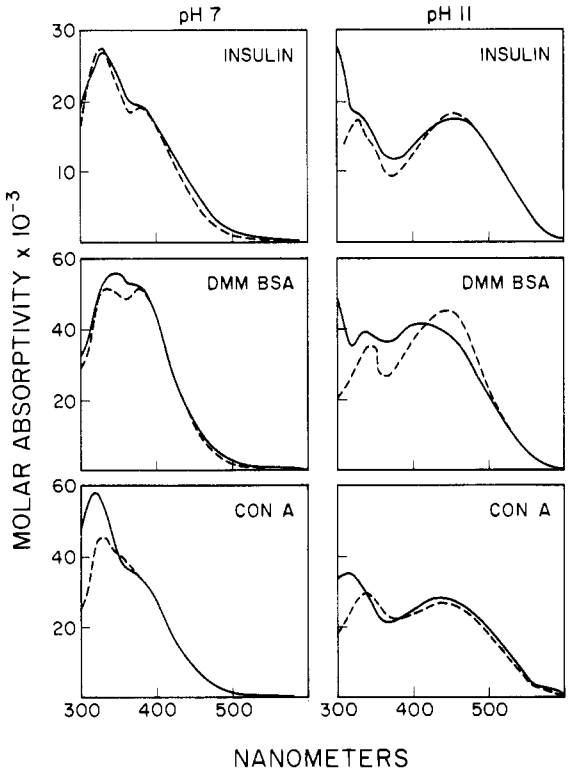

FIGURE 1: Comparison of sulfanilazo protein spectra (solid curves) and their simulations (dashed curves) for three proteins at pH 7 (left panels) and 11 (right panels). Proteins were azo coupled as described in footnotes *a* of Tables I and II. The concentrations of the mono(sulfanilazo) amino acids used in the simulations were calculated from the appropriate data in Tables I and II. DMMBSA is BSA that was acylated with dimethylmaleic anhydride prior to azo coupling (third entry from top of Table II).

suggestion that lack of excellent fit is due to a small microenvironmental effect in this case rather than a failure of spectroscopic method comes from two observations: (1) The amount of azo coupling at the C-4 and C-2 positions of histidine is calculated from the pH 7 azo protein spectrum, and the agreement between the azo protein and its simulation at pH 7 is quite good. (2) A better fit at pH 11 was obtained by using the N^{α}-acetyl-2-mono(sulfanilazo)-L-histidine spectrum at a pH slightly below 11 (data not shown). This is equivalent to increasing the pK_a of the C-2 derivative in the protein.

It is very important to note that while a shift in pK_a of the imidazole/imidazolate equilibrium of the monoazohistidines will affect the pH 11 spectrum of the azo protein, this shift will not affect the determination of the amount of the individual monoazohistidines. These quantities are obtained from the pH 7 spectrum (steps F and G), well removed from the pK_a values of the monoazohistidines [see Table III in Pielak et al. (1984)].[4]

The results of the simulation experiments indicate that the visible spectral method is valid and that there is no appreciable formation of bisazo derivatives of tyrosine or histidine. The bisazo derivatives absorb strongly between 300 and 600 nm (Pielak et al., 1984) and would, if present, preclude the excellent simulations obtained with only the monoazotyrosine and -histidine model compounds.

^{35}S Incorporations. Total incorporated [^{35}S]sulfanilic acid was compared to the number of tyrosine and histidine residues detected by spectral analysis (Table I). It is evident from Table I that for each protein investigated, amino acids were modified that were not detected by spectral analysis. These are referred to as nonspectrally determined modifications (vide infra).

Modification of Free-Amine Groups in Proteins. It has been reported that diazonium salts react with primary amines (Burton & Waley, 1967; Gelewitz et al., 1954; Higgins & Harrington, 1959; Howard & Wild, 1957; Kagan & Vallee, 1969; Malinowski & Fridovich, 1979; Takenaka, 1969). In every protein studied, the number of nonspectrally detected modifications was reduced or eliminated upon acylation of free amines (N-termini, lysines) prior to azo coupling (compare Tables I and II). Note that for BSA, even a large excess of $(AcO)_2$ does not decrease the number of nonspectrally determined modifications below one. This is probably due to the reaction of the one free sulfhydryl in BSA with the diazonium salt (vide infra). None of the other proteins in Table I contain free sulfhydryls. Also, it was found that the number of TNBS-reactive amines lost upon azo coupling of either RNase or insulin was equal to the number of nonspectrally detected modifications. TNBS was also used to establish that reaction of diazotized arsanilic acid with proteins results in free-amine modification (data not shown). These data suggest that lysine modification accounts for most of the incorporated diazonium salt not detected by spectral analysis.

While acylation decreases or eliminates azo coupling of free amines, it also changes the susceptibility of tyrosine and histidine residues to azo coupling (compare Tables I and II).

[4] It is unlikely that microenvironmental effects will be seen in the case of tyrosine modification since the pK_a values of mono(arsanilazo)- and mono(sulfanilazo)tyrosine in water are 9.4 and 9.6, respectively (Pielak et al., 1984) and a very large change in pK_a would be required to change their spectral properties at either pH 7 or 11 (the pH values used for spectral analysis).

Table II: Spectral Analysis and ^{35}S Incorporation Studies of Various Sulfanilazo Acylated Proteins

protein[a]	anhydride[b]	anhydride/protein[c]	stoichiometry of mono(sulfanilazo) amino acids from spectral analysis				stoichiometry of azo amino acids (^{35}S)	stoichiometry of azo amino acids not detected by spectral analysis
			azotyrosine	azohistidines		total		
				C-2	C-4			
BSA	$(AcO)_2$	210	1.10	0.28	0.70	2.08	3.88	1.80
	$(AcO)_2$	1300	1.28	1.05	0.49	2.82	4.05	1.23
	DMMA	980	1.90	1.41	0.27	3.58	4.65	1.07
insulin	$(AcO)_2$	210	1.70	0.38	0.00	2.08	2.06	-0.02
	DMMA	150	2.54	0.57	0.00	3.11	3.37	0.26
RNase	$(AcO)_2$	210	1.25	0.20	0.06	1.51	2.29	0.78
	DMMA	110	1.71	0.15	0.01	1.87	2.28	0.41
carbonic anhydrase A	DMMA	150	1.08	1.02	0.55	1.65	3.00	0.35

[a] All acylated proteins were reacted with a 5-fold excess of diazotized [^{35}S]sulfanilic acid in 0.02 M borate–0.5 M NaCl, pH 9.0. See Experimental Procedures for details. [b] Acetic anhydride, $(AcO)_2$; dimethylmaleic anhydride (DMMA). [c] Ratio of anhydride to protein used in the prior acylations. See Experimental Procedures for details.

Table III: Spectral Analysis of Various Arsanilazo Proteins

protein[a]	stoichiometry of mono(arsanilazo) amino acids from spectral analysis			
	azotyrosines	azohistidines		total
		C-2	C-4	
BSA	0.18	0.32	0.00	0.50
insulin	0.24	0.05	0.01	0.30
RNase	0.37	0.07	0.03	0.47
α-chymotrypsin	0.28	0.00	0.02	0.30
carbonic anhydrase B	0.34	0.27	0.15	0.76
carboxypeptidase A	0.79	0.00	0.01	0.80

[a] All proteins were coupled with a 5-fold excess of diazotized arsanilic acid at pH 9.0 in 0.02 M borate–0.5 M NaCl. See Experimental Procedures for details.

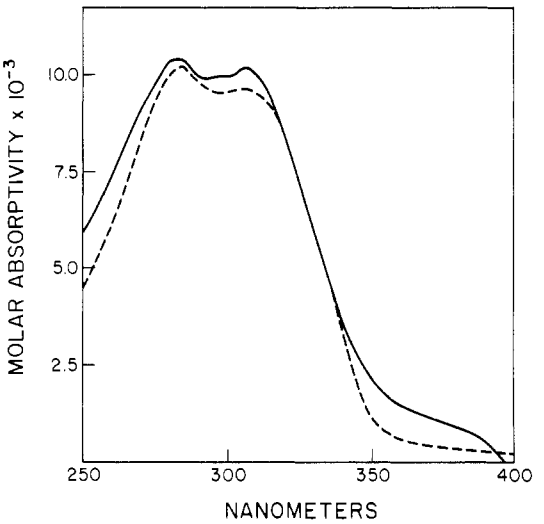

FIGURE 2: Spectrum of unstable azo amino acids in sulfanilazo-RNase (solid curve) and spectrum of the purified product from the reaction of an excess of poly(L-lysine) with diazotized [^{35}S]sulfanilic acid (dashed curve). The protein spectrum was obtained as described in the text. The other spectrum was obtained by reacting a solution of poly(L-lysine) (1.56×10^{-2} M monomer) with 5×10^{-4} M diazotized [^{35}S]sulfanilic acid in 0.02 M borate–0.5 M NaCl, pH 9.0, for 1 h and then preparing a 300-μL sample for spectral analysis at pH 7 (as described under Experimental Procedures) and recording the spectrum immediately. The molar absorptivity for the poly(L-lysine) derivative is based on the amount of radioactivity in the sample used to obtain the spectrum.

The changes in reactivity can be rationalized in terms of alteration of protein charge brought about by acylation (Pielak, 1983).

Products Formed upon Reaction of Diazonium Salts with Primary Amines. The reaction of diazotized sulfanilic acid with primary amines has been reported to result in a product with an absorbance maximum at 363 nm (Higgins & Harrington, 1959). Similarly, we have found that the reaction of poly(L-lysine) with a 10-fold excess of diazotized sulfanilic acid (excess with respect to the amount of ε-amine) gives rises to a species with an absorbance maximum at 354 nm (product I). However, if the poly(L-lysine) is modified with 0.1 molar equiv of diazonium salt, then a species characterized by 284- and 307-nm maxima predominates (product II, see Figure 2). Although both species are stable for several days at pH 9, they decompose rapidly at pH 7 or below.

It is likely that product I is a 3-alkyl-1,5-diarylpentadiazene and that product II is a 1-alkyl-3-aryltriazene. These species are generated by azo coupling of 2 and 1 equiv of diazonium salt/equiv of primary amine, respectively. That the pentadiazene is in facile equilibrium with triazene plus free di-

azonium salt is supported by the observations that exclusion chromatography converts the pentadiazene of poly(L-lysine) into the excluded triazene plus retained diazonium salt and that addition of phenol to pentadiazene solutions results in the formation of azophenol. Addition of phenol to a solution of triazene results in no reaction.

In this study, all azo protein reaction mixtures are quenched with phenol *and* passed over a gel-exclusion column before any properties are determined. Therefore, pentadiazenes are not observed. It is also important to note that the triazene has a molar absorptivity of less than 1000 at 410 nm, pH 7, which decreases to 0 at 540 nm, pH 11. Therefore, triazene formation in azo proteins will not interfere with the determination of monoazotyrosine and -histidines by spectral analysis. The spectral changes observed for freshly prepared azo-coupled RNase illustrate this behavior. The absorbance of sulfanilazo-RNase (2×10^{-5} M) at pH 7 was observed to decrease between 250 and 380 nm as a function of time with a half-life of about 30 min. The rate was a function of pH: fast at pH 7, negligible at pH 11. Using diazotized [^{35}S]sulfanilic acid, it was noted that while the total sulfanilic acid incorporated decreased, the number and type of azotyrosines and -histidines remained constant. In order to obtain a spectrum of the unstable product, the pH 7 spectrum recorded after the absorbance stopped decreasing was subtracted from the spectrum recorded immediately after quenching and purification (Figure 2). The molar absorptivity was estimated by normalizing to the protein concentration and dividing by the number of [^{35}S]sulfanilic acids lost. The loss of absorbance described above was observed for most of the azo proteins containing azoamine modification, but the rate and its pH dependence varied widely.

The question of the decomposition products was addressed. TNBS reactions indicated that the two free amines lost upon azo coupling of RNase did not return after decomposition. White & Scherrer (1961) have reported that primary 1-alkyl-3-aryltriazenes are susceptible to nucleophilic attack after protonation of N_3. In aqueous solution, therefore, the triazenes should react to form the corresponding primary alcohols. Amino acid analysis of sulfanilazo-RNase indicated that lysine was lost and α-amino-ϵ-hydroxycaproic acid was formed upon decomposition, while in (sulfanilazo)insulin, loss of glycine and phenylalanine (the N-terminal amino acids) was observed (Pielak, 1983).

Reaction of Diazonium Salts with N^{α}-Acetyl-L-arginine, -L-proline, and -abrine. Higgins & Harrington (1959) reported that the diazonium salt of sulfanilic acid reacts with arginine to produce a species absorbing at about 360 nm. It is likely that these authors observed the reaction of the free α-amine since in this study no reaction was observed with the N^{α}-acetyl derivative.

These authors also reported that diazonium salts react with the secondary amine of proline. The product of the reaction is the secondary 1-alkyl-3-aryltriazene as evidenced by its near-UV spectrum. (Secondary amines cannot form pentadiazenes.) Upon treatment with acid, the secondary amine re-forms. It was also shown that at pH 9.0 aryl diazonium salts do not react with the indole portion of abrine (N^{α}-methyl-L-tryptophan). However, in acid solution indole has been shown to react with 3-diazonium 1,2,4-triazole (DeTraglia et al., 1979).

Comparison of Arsanilazo with Sulfanilazo Derivatives. By comparing Tables I and III, it can be seen that the negatively charged diazotized arsanilic acid is significantly more reactive than the zwitterionic diazotized sulfanilic acid. The difference in reactivity is most likely attributable to the charge difference.

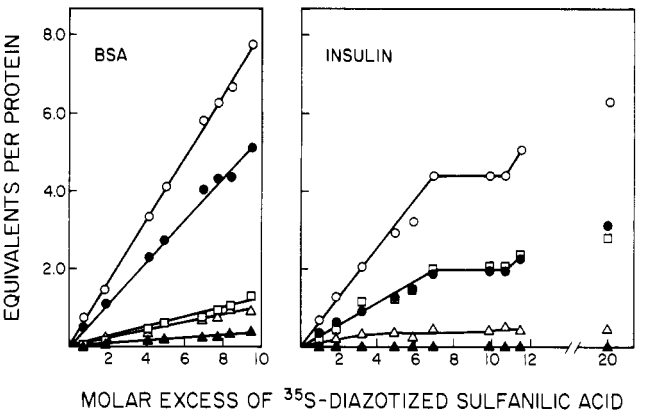

FIGURE 3: Degree of tyrosine, histidine, and nonspectrally determined modification in BSA (left panel) and insulin (right panel) as a function of diazotized [^{35}S]sulfanilic acid concentration. The reactions were carried out with 0.1 mM protein in 0.02 M borate–0.5 M NaCl, pH 9.0, at the concentrations of diazotized [^{35}S]sulfanilic acid indicated. The stoichiometries of mono(sulfanilazo)tyrosine (□), 2-mono(sulfanilazo)histidine (△), and 4-mono(sulfanilazo)histidine (▲) were determined from the sulfanilazo protein spectra with the appropriate equations as described under Experimental Procedures. The number of nonspectrally determined modifications (●) is simply the difference between the total number of spectrally detected azo amino acids and the total amount of ^{35}S incorporated (○).

Dependence of Azo Coupling on the Diazotized Sulfanilic Acid Concentration. For BSA, the degree of modification increased linearly with increase in diazonium salt concentration (Figure 3). The number of nonspectrally detected modifications always exceeds the sum of the other three azo-coupled residues, reflecting not only the ratio of lysine to tyrosine and histidine in BSA but also the inherent reactivity of free sulfhydryls (vide infra). The linear behavior indicates that BSA has an excess of potentially reactive residues with respect to the range of diazonium salt concentration studied.

In contrast to BSA, insulin exhibits selective reactivity in terms of both type and number of reactive residues. Throughout the range of diazonium salt concentrations studied, minimal modification of the two histidine residues was observed. While insulin contains four tyrosines, only two are modified from a ratio of about 8 to 1 to a ratio of 11 to 1 (reagent to protein). When the ratio is increased significantly (20:1), further tyrosine modification is detected (Figure 3). This behavior suggests specific modification. The modification of free amines (two N-terminal and one lysine) follows a pattern similar to tyrosine modification except that at a ratio of 20:1, all the amines are modified.

Dependence of Azo Coupling on pH. The reactivity of BSA and thermolysin was studied as a function of pH at a ratio of diazonium salt to protein of 5 to 1 (Figure 4). For BSA, the reactivity pattern at a given pH is comparable to that observed for BSA as a function of diazonium salt concentration (Figure 3). The enhanced reactivity with increased pH is probably not due to the increased ionization of the amino acid side chains since the pK_as of the modifiable side chains are outside of the pH range studied. The enhancement of free-amine modification may reflect in part the ionization of the N-terminus (p$K_a \sim 8$), but it will be noted that a total of three nonspectrally determined modifications occurs over the pH range studied. However, in some proteins abnormal pK_a values of specific side chains may result in unusual pH-dependent reactivity. The one modified residue in BSA at pH 7 most likely represents reaction of the one free sulfhydryl in the protein (vide infra).

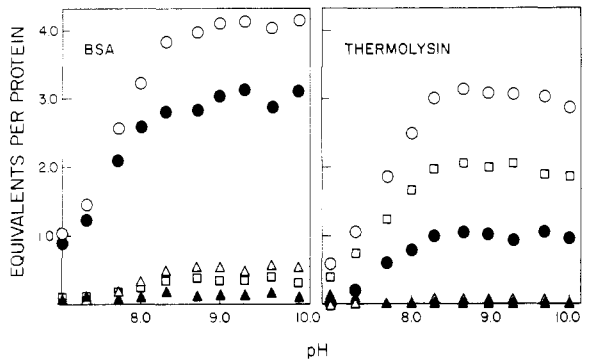

FIGURE 4: Degree of tyrosine, histidine, and nonspectrally determined modifications in BSA (left panel) and thermolysin (right panel) as a function of pH. The modifications were carried out as described in the text in 0.02 M phosphate, 0.02 M veronal, 0.02 M borate, and 0.5 M NaCl at the pH values indicated. The explanation of the symbols is given in the legend to Figure 3.

The dominant factor controlling the increased reactivity of BSA with increased pH is very likely the increased reactivity of the diazonium salt itself. From pH 8.3 to 10, the reactivity has reached nearly a maximum value. It could be argued that the plateau region for BSA in Figure 4 represents a specific set of reactive residues. However, inspection of Figure 3 shows that at pH 9 the reactivity increases with the ratio of diazonium salt to BSA.

In contrast to the behavior of all other proteins investigated, tyrosine modification exceeds lysine modification in thermolysin (Figure 4). Thermolysin is the only protein in this study in which the number of tyrosines greatly exceeds the number of lysines (Titani et al., 1972). The reactivity profile of thermolysin between pH 7 and 10 suggests modification of two specific tyrosines. None of the eight histidine residues were modified.

Reaction of Free Sulfhydryls with Diazonium Salt. YADH was reacted with a 5-fold molar excess (with respect to monomer) of either diazotized [^{35}S]sulfanilic acid or diazotized arsanilic acid at pH 9.0 (0.02 M borate–0.5 M NaCl) and pH 6.8 (0.5 M phosphate). Less than 0.1 spectrally detectable residue was found per monomer in all cases. However 4.01 and 1.80 mol of ^{35}S per monomer were incorporated at pH 9 and 6.8, respectively. Using DTNB, it was shown that sulfhydryls were lost upon azo coupling at pH 9.

The spectrum of the YADH derivative had one absorption maximum at 325 nm that was pH independent. The derivative was stable for at least 48 h at pH 7. These spectral characteristics are similar to those reported by Sogin & Plapp (1976) for the reaction of liver alcohol dehydrogenase with diazonium 1*H*-tetrazole. These authors concluded that only sulfhydryls were modified. The supposed azo-coupled sulfhydryl spectrum in azo-YADH was obtained by subtracting the spectrum of native YADH from that of the sulfanilazo protein derivative (under identical conditions) and was compared to the spectrum of the model compound obtained by reacting N^{α}-acetylcysteine with an equal molar amount of diazotized sulfanilic acid under conditions identical with those used to modify the protein. The spectra overlay (Figure 5), leaving little doubt that sulfhydryls are modified in azo-YADH. Very similar spectra have been reported for the reaction of aldolase with diazotized *p*-aminobenzoic acid (Felicioli et al., 1975) and sulfanilic acid (Burton & Waley, 1967). Since the molar absorptivity of the model compound is less than 1500 at pH 7 and 380 nm, less than 700 at pH 7 and 410 nm, and 0 at pH 11 and 540 nm, azo coupling of sulfhydryls, like free amines, will not interfere with spectral analysis.

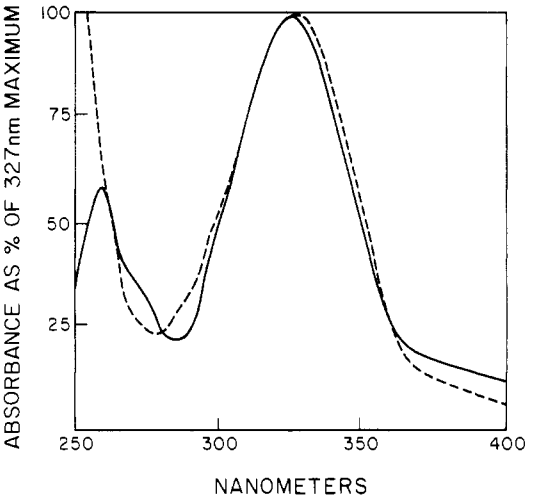

FIGURE 5: Normalized spectrum of modified residues in sulfanilazo-YADH (solid curve) and normalized spectrum of reaction mixture containing *N*-acetylcysteine (10 mM) plus diazotized sulfanilic acid (0.5 mM) in 0.02 M borate–0.5 M NaCl, pH 9.0, after a 1 to 10 dilution in the same buffer. See text for details.

Small, but significant, amounts of spectrally detectable modification could only be obtained by reaction of YADH with a large excess of iodoacetic acid in sodium dodecyl sulfate, prior to azo coupling. Even under these conditions significant azo coupling of sulfhydryl groups occurred. For BSA, even a large excess of acylating reagent does not block modification of one nonspectrally determined residue (compare Tables I and II). It is likely that this is the one free sulfhydryl in BSA. Further evidence for reaction of this residue in BSA is seen in Figure 4, where one nonspectrally determined modification was observed at pH 7. The reactivity of histidine, tyrosine, and free amino residues can be controlled by changing the pH from 7 to 10. However, free-sulfhydryl modification is independent of pH in this range as observed for YADH.

Summary and Conclusions

The spectral method presented permits the accurate and rapid determination of the extent of histidine and tyrosine azo coupling in proteins and can discriminate between the two different structural isomers of monoazohistidine. Bisazo derivatives are not formed in detectable quantities under the conditions used. While significant amounts of 4-monoazohistidine are formed in model reactions (Pielak et al., 1984), in general, much less C-4 than C-2 modification occurs in azo proteins. This behavior, as well as the apparent lack of formation of bisazo derivatives in proteins, is most likely due to steric constraints. Although microenvironmental effects are sometimes seen in the case of azohistidine, it appears they do not adversely affect the reliability of the spectral method.

For the azo-coupled proteins, it was not possible to account for the total amount of modification by spectral analysis. These nonspectrally determined azo amino acids represent the reaction of free-amine groups with the diazonium salts to form triazenes, which breakdown to the corresponding alcohols (Pielak, 1983). Azo coupling of amines can be protected by acylation. It has also been shown that sulfhydryl groups react with diazonium salts to form a stable derivative with a pH-independent UV spectrum. Neither of these modifications interfere with the determination of azotyrosine and -histidine in azo proteins by spectral analysis. The extent of amine and sulfhydryl modifications can be assessed by TNBS and DTNB, respectively.

In general, protein reactivity to azo coupling can be enhanced by increasing diazonium salt concentration and pH.

Tyrosine is more reactive than histidine in nearly every protein studied. For several of the proteins, the data obtained were consistent with specific modification. Changes in the reaction pH, the excess of diazonium salt, the type of diazonium salt, and chemical modification prior to azo coupling can be used to differentially affect the type and number of residues modified.

The fact that free amines represent the majority of residues modified in all but one of the azo proteins studied is significant in terms of using diazonium salts as spectral probes for structure–function relationships since these modifications are not easily detected by spectral analysis. The instability of the triazenes may also explain the low net transfer of protein from polyacrylamide or agarose gels to diazobenzyloxymethyl paper (Renart et al., 1979).

Registry No. Insulin, 9004-10-8; Con A, 11028-71-0; RNase, 9001-99-4; α-chymotrypsin, 9004-07-3; carbonic anhydrase, 9001-03-0; thermolysin, 9073-78-3; carboxypeptidase A, 11075-17-5; mono(sulfanilazo)tyrosine, 37019-59-3; mono(sulfanilazo)histidine (C-2), 88180-54-5; mono(sulfanilazo)histidine (C-4), 88180-55-6; $(AcO)_2$, 108-24-7; DMMA, 766-39-2; mono(arsanilazo)tyrosine, 33650-94-1; mono(arsanilazo)histidine (C-2), 88180-56-7; mono(arsanilazo)-histidine (C-4), 88180-57-8; diazotized [^{35}S]sulfanilic acid chloride, 88180-58-9; diazotized arsanilic acid chloride, 40847-26-5; alcohol dehydrogenase, 9031-72-5; poly(L-lysine) (homopolymer), 25104-18-1; poly(L-lysine) (SRU), 38000-06-5; L-lysine, 56-87-1; N^{α}-acetylcysteine, 616-91-1.

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