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Conformational Aspects of Polypeptide Structure. XXVIII. Side-Chain Cotton Effect from Poly-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine*

Ettore Benedetti and Murray Goodman

ABSTRACT: Quantitative diazotization of poly-L-*p*-aminophenylalanine, followed by coupling of this unisolated intermediate with *p*-cresol, leads to poly-L-*p*-(5′-hydroxy-2′-methylphenylazo)phenylalanine. Conformational analysis of this azoaromatic polypeptide was carried out in hexafluoro-2-propanol, dimethylacetamide, and trifluoroacetic acid. In the first two solvents, which we generally considered helix supporting, side-chain Cotton effects are detected, while for the third solvent no ellipticity band is observed. We point out that macromolecules with bulky side chains may not be able to assume a completely random structure because steric interactions lead to specific preferred conformations. The resulting level of order or occasional periodicity in the main chain or in the side chains or in both produces the observed Cotton effects.

For most polymers derived from α-amino acids containing strongly absorbing side-chain chromophores such as imidazole, indole, or aromatic groups, unique assignment of secondary structure in solution is difficult (Goodman *et al.*, 1968). The difficulties arise mainly from overlapping of the optically active electronic transitions of the side chain with the transitions from the main-chain amide chromophores. On the other hand, amino acid aromatic side chains substantially affect the conformation of poly-α-amino acids and proteins in solution (Goodman and Toniolo, 1968). Their structure and conformation are in part determined by electronic and steric interactions among side-chain chromophores and between the side chains and the optically active centers in the polypeptide main chain. Optical methods such as optical rotatory dispersion or circular dichroism are powerful tools to yield information on side-chain structure and over-all conformational relationship in poly-α-amino acids and naturally occurring materials.

Several aromatic amino acids containing auxochromic substituents in the side chains have been investigated in our laboratories. We recently reported on the synthesis and conformational characterization of two azoaromatic polypeptides: poly-L-*p*-(phenylazo)phenylalanine (Goodman and Kossoy, 1966) and poly-L-*p*-(*p*′-hydroxyphenylazo)phenylalanine (Goodman and Benedetti, 1968). In the present paper we report on the synthesis and conformational analysis of a new azoaromatic poly-

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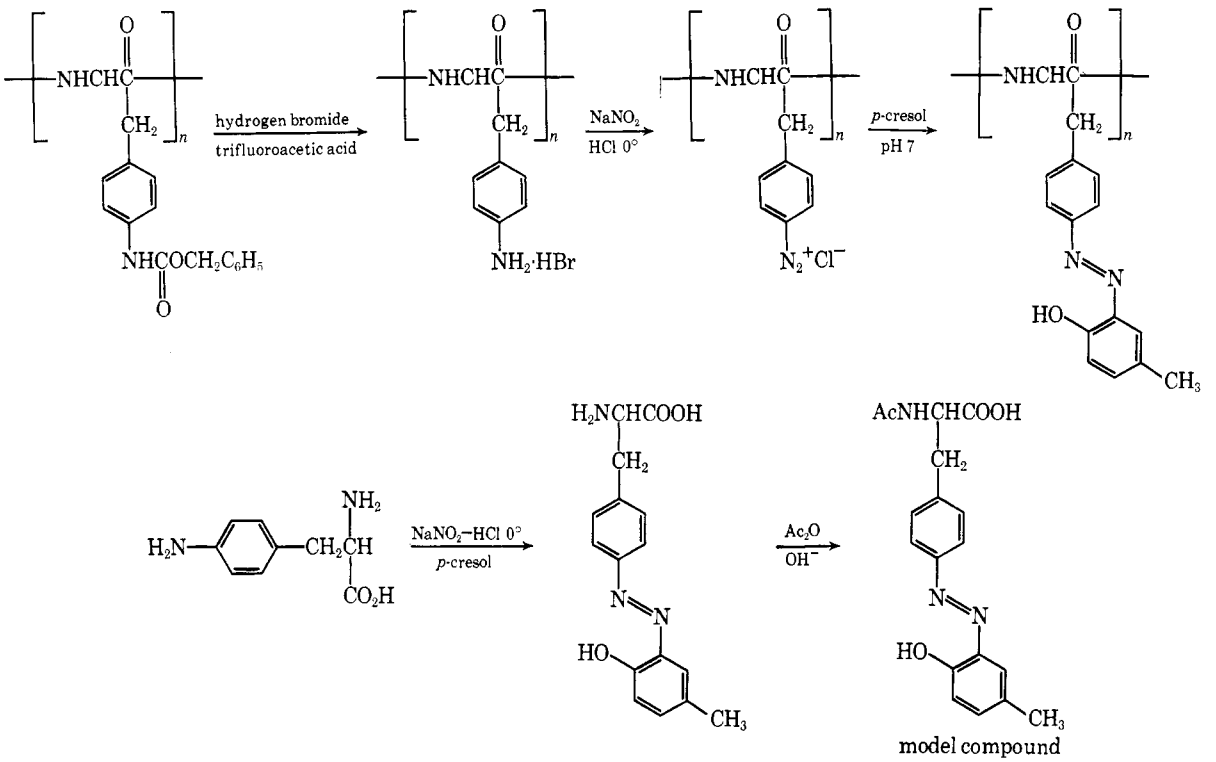
SCHEME I

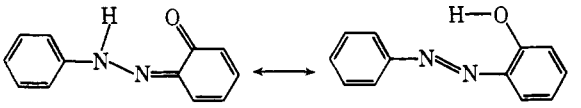


α-amino acid, L-*p*-(2′-hydroxy-5′-methylphenylazo)-phenylalanine, in different solvent media dimethylacetamide, hexafluoro-2-propanol, and trifluoroacetic acid. We compare results on the polymeric system with a model compound, *N*-acetyl-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine.

Results and Discussion

Synthesis. The synthesis of the desired azoaromatic polypeptide is outlined in Scheme I. *N*-Carbobenzoxylation of L-*p*-aminophenylalanine gives the dicarbobenzoxy derivative which is treated with phosphorus pentachloride to obtain the corresponding *N*-carboxyanhydride (Katchalski and Sela, 1953). Poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine is obtained by polymerization in dry tetrahydrofuran using sodium methoxide as the initiator (Blout and Karlson, 1956). Subsequent removal of the N^{ω}-carbobenzoxy group leads to poly-L-*p*-aminophenylalanine, which is diazotized and coupled with *p*-cresol to yield the desired polypeptide, poly-L-*p*-(2′-hydroxy-5′methylphenylazo)phenylalanine. The model compound, *N*-acetyl-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine, is obtained by diazotization and coupling of L-*p*-aminophenylalanine with *p*-cresol followed by acetylation of the free α-amino acid.

Many authors have been interested in *o*-hydroxyazobenzene and its derivative from the spectroscopic point of view in order to establish the presence of tautomerization for such materials. Ospenson (1951) studied the ultraviolet and visible spectra of *o*-hydroxyazobenzene and derivatives. He interpreted these spectra as a proof for the existence of the hydrazonic form.



However Burawoy *et al.* (1952) arrived at the conclusion that a true azo compound exists in solution. He based his interpretation on the complete absence of any solvent effect in the absorption spectrum of *o*-hydroxyazobenzene. The latter structural assignment is supported by Wettermark *et al.* (1965) and more recently by Gabor *et al.* (1967), since a tautomeric equilibrium may be expected to depend upon the polarity of the solvent, with polar solvents favoring the more polar hydrazonic tautomer. Results from nuclear magnetic resonance studies and the lack of luminescence for these compounds can be cited as evidence that they exist mainly as azo tautomers. In the solid state, infrared and electronic spectra indicate that *o*- and *p*-hydroxyazobenzene exist as the azo tautomers (Hadzi, 1956). Nuclear magnetic resonance studies in CCl_4 solutions of 2-hydroxyazobenzene and some derivatives show that strong internal hydrogen bonds are observed (Reeves, 1960). The latter observation was suggested a long time ago (Oddo and Puxeddu, 1906) and many hypotheses on the formation and the nature of a coplanar six-membered ring have been put forth. The presence of an internal hydrogen bond in *o*-hydroxyazoaromatic com-

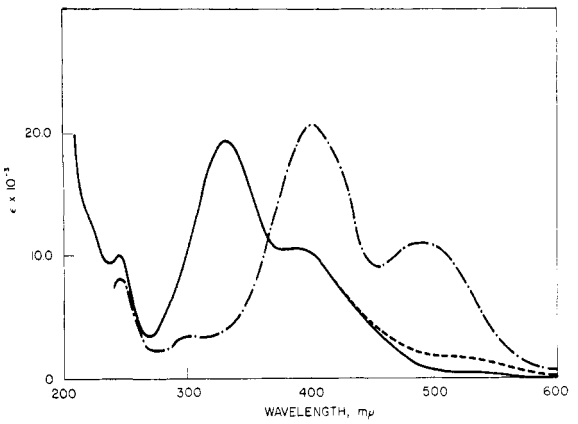
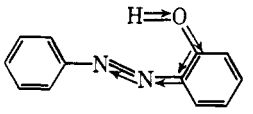


FIGURE 1: Ultraviolet and visible spectra of poly-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine in dimethylacetamide (———), hexafluoro-2-propanol (·····), and trifluoroacetic acid (-·-·-).

pounds is also supported by their low degree of association in organic solvent as compared with *p*-hydroxyazoaromatic compounds, their densities, surface tensions, and infrared spectra. Internal hydrogen bonds are essentially electrostatic and involve polarizations of the entire molecule as indicated.



These effects can be responsible for the small displacements to longer wavelengths of bands arising from electronic transitions involving π orbitals of phenolic substances containing an internal hydrogen bond (Morton and Stubb, 1940). According to a more recent hypothesis (Shigorin, 1953, 1959; Nurmukhametov *et al.*, 1961) the hydrogen bond in molecules with a conjugated system of bonds is formed as a result of three kinds of interactions: dipole–dipole (E_d), acceptor–donor (E_a), and π-electron (E_π). $E_H = E_d + E_a + E_\pi$. In conjugated systems, π-electron interactions play the determining role in the formation of the hydrogen bond, especially in the excited state of the molecule. However π-electrons become important only when the ring formed by means of hydrogen bonding is coplanar with the remainder of the molecule.

Exactly such a plane, "quasi-aromatic," six-membered ring is found in *o*-hydroxyazoaromatic compounds; it is closed by a hydrogen bond and is coplanar with the remainder of the molecule.

The planar character of this structure for such compounds is confirmed by the value of their dipole moments.

Wettermark *et al.* (1965) found that *o*-hydroxyazobenzene and 2-hydroxy-5-methylazobenzene yield the same ultraviolet and visible spectra in several solvents of different polarity. As a result, these workers concluded that these compounds exist in solution as true phenols. We assumed from these interpretations that the side chains of the azoaromatic polypeptide, the 2-hydroxy-5-methyl azoaromatic groups, exist as azo rather than hydrazonic tautomers. In addition, an intramolecular hydrogen bond (forming a quasi-aromatic six-membered coplanar ring) is present which requires a *trans* configuration with respect to the N=N bond.

The ultraviolet and visible spectra of the poly-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine in dimethylacetamide, hexafluoro-2-propanol, and trifluoroacetic acid are shown in Figure 1. From these spectra the question of the extent of diazotization and coupling can be answered. The close similarity in shape and magnitude of the bands among the *o*-hydroxyazobenzene, the 2-hydroxy-2-methylazobenzene, our model compound, and the polymer in question enables us to establish that nearly 100% of the amino groups of the poly-L-*p*-aminophenylalanine were converted into the azoaromatic side-chain groupings. From the comparison of the values of the molar extinction coefficients (Table I) for the three major electronic transitions in the ultraviolet spectra of the polymer and the model compound we do not observe hypochromic effects. For azoaromatic polypeptides with *p*-hydroxyl substitution we detect a large conformationally dependent hypochromicity. Unfortunately no assignments of these electronic transitions are available in the literature. For azobenzene and its derivative all the major electronic transitions have been assigned. Of course, the high degree of symmetry along the lengthwise axis of the azobenzene and its *para* derivatives is destroyed by any substituent in *meta* or *ortho* position. This results in complications and mixing of energy levels which can lead to difficulties in the assignments of the major bands. The absorption spectra of the polymer in dimethylacetamide and hexafluoro-2-propanol are quite similar; the only difference occurs above 480 mμ where the hexafluoro-2-propanol solution exhibits a greater absorption. This may be respon-

TABLE I: Ultraviolet and Visible Spectra of the Polymer and the Model Compounds.

Solvent	Polymer (mμ)	Model Compound (mμ)
Dimethylacetamide	385 (4.02)	383 (3.99)
	332 (4.29)	320 (4.29)
		244 (4.02)
Hexafluoro-2-propanol	510 (3.28)[a]	384 (3.99)
	385 (4.02)	320 (4.30)
	332 (4.29)	244 (4.02)
Trifluoroacetic acid	490 (4.04)	490 (4.05)
	420 (4.24)[a]	403 (4.33)
	403 (4.31)	310 (3.56)
	310 (3.51)[a]	244 (3.97)
	245 (3.92)	

[a] Shoulder.

sible for the red color of these solutions. On the other hand, in the very acidic solvent trifluoroacetic acid which is capable of protonating the azoaromatic groups the entire spectrum seems to be shifted to a longer wavelength with greater separation of the major bands which occur at 490, 402, and 246 mμ. The latter band (246 and 244 mμ for polymer solutions, respectively, in trifluoroacetic acid and hexafluoro-2-propanol or dimethylacetamide and 244 mμ for *o*-hydroxyazobenzene solutions) does not show any dependence upon change of solvent. Tentatively this transition involves pure aromatic orbitals localized on the phenyl rings such as ϕ orbitals, while the other two bands at longer wavelengths must involve orbitals localized on the azo groups and atoms close to this group, especially the hydroxyl function in *ortho* positions.

Circular Dichroism Spectra. The circular dichroism spectra of the polyazoaromatic peptide in dimethylacetamide and hexafluoro-2-propanol are shown in Figure 2. In the case of the hexafluoro-2-propanol solution the spectrum exhibits a peak centered at 358 mμ and a trough at 332 mμ with a crossover at 333 mμ, all in the absorption region of the azoaromatic chromophore. In the peptide chromophoric region below 230 mμ, only a huge single trough ($[\theta] = -38{,}500$) centered at 197 mμ is present. The existence of one single band in this region seems to demonstrate that the polypeptide assumes a random coil conformation in this solvent (Holzwarth and Doty, 1965). The intensity of the band and its position at 197 mμ are only 6 mμ displaced from the predicted 191-mμ π–π^* spectral transition for the peptide group when the polymer conformation is a random coil. However we must emphasize our contention that macromolecules containing extremely bulky side chains cannot assume a completely random structure because steric hindrance will lead to specific conformational preferences. This bias can establish some ordering or occasional periodicity in the main chain, in the side chain of a polymer, or in a combination of both (Bradley *et al.*, 1966). As a matter of fact, the polyazoaromatic peptide in hexafluoro-2-propanol shows side-chain Cotton effects which indicate that the bulky azobenzene residues must arrange themselves with some definite periodical order of unknown extent. The presence of an additional six-membered quasi-aromatic ring contributes to the steric hindrance of the side chains which produce the above-mentioned order. We have reported (Benedetti *et al.*, 1968) conformational changes for poly-L-*p*-(*p'*-hydroxyphenylazo)phenylalanine in trimethyl phosphate–trifluoroacetic acid solvent systems. We found evidence for right-handed α helices when the premixed solvent composition contains no more than 10% trifluoroacetic acid. However we found a completely different order for the bulky azoaromatic side chains and an apparent absence of main-chain order if trifluoroacetic acid was allowed to interact extensively with the polypeptide solute. The latter conclusion was based on the presence of azoaromatic side-chain Cotton effects and on the absence of any dichroic band in the peptide spectral region.

In the case of solutions of the polypeptide in dimethylacetamide, the circular dichroism spectrum (Figure 2)

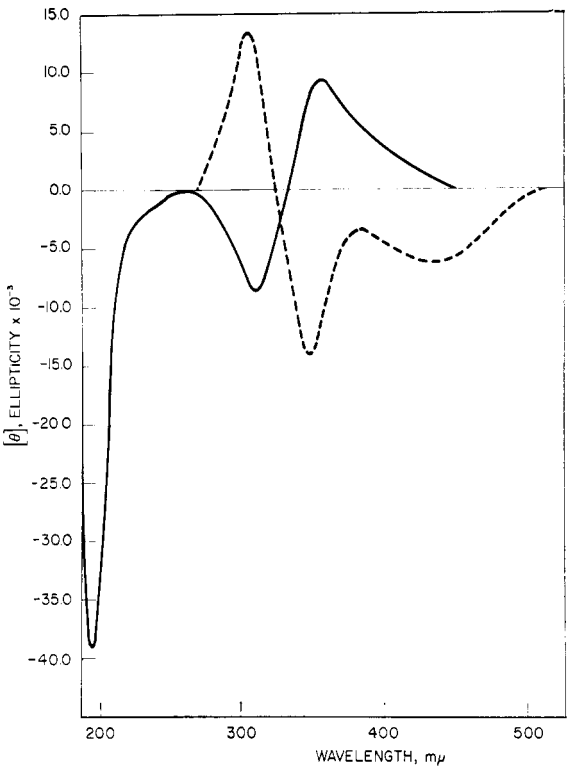


FIGURE 2: Circular dichroism spectra of poly-L-*p*-(2'-hydroxy-5'-methylphenylazo)phenylalanine in dimethylacetamide (---------) and hexafluoro-2-propanol (———).

exhibits two troughs at 433 and 348 mμ and a peak at 398 mμ with a crossover at 308 mμ. Further penetration into the shorter wavelength ultraviolet region is impossible because of solvent cut-off (250 mμ). The polymer shows azoaromatic Cotton effects of opposite sign of ellipticity as compared with our results in hexafluoro-2-propanol. No assignment for the over-all conformation is possible. Furthermore, it is unknown if the different shapes of the circular dichroism spectra in hexafluoro-2-propanol and dimethylacetamide are due to the fact that the polymer can have side-chain order with two screw senses. However this is a dangerous assumption since Tinoco (1964) pointed out that very small changes in transition dipole moments can lead to Cotton effects of opposite sign. In addition, the observed bands of opposite sign with very nearly the same intensity probably mean that the coupled transition dipoles interact so that the absorption band is split into two bands assuming that only one transition is responsible for the observed circular dichroism spectrum. The circular dichroism spectra of the model compound in both solvents (hexafluoro-2-propanol and dimethylacetamide) do not show any detectable band over the range of the azoaromatic electronic transitions. In the peptide absorption region only a weak positive dichroism is detectable. We must point out the difficulties in establishing exact rotation values for the model compound where the rotations are small and the ultraviolet absorption bands are intense.

In trifluoroacetic acid, a strongly hydrogen-bonding solvent, we observe no dichroic band over the range

600–240 mμ for both the polymer and the model compound. This is a significant result which demonstrates that when the azoaromatic side chains are protonated the internal hydrogen bonding and the quasi-aromatic six-membered ring are disrupted. The polymer side chains probably lose all ordering and the polymer assumes a true random-coil structure. This result supports our thesis that in dimethylacetamide and hexafluoro-2-propanol order is achieved among the side chains.

Conclusion

We have shown that we can quantitatively diazotize poly-L-*p*-aminophenylalanine and that this unisolated intermediate can be allowed to couple completely with *p*-cresol. In this manner we prepared poly-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine. We were unable to dissolve the polymer in aqueous base because the *o*-hydroxyazoaromatic group is an extremely weak acid ($pK_a = >10$). We were able to study the ultraviolet and circular dichroism spectra in three solvents. In dimethylacetamide we observed ultraviolet bands and Cotton effects attributable to ordered side chains. With this solvent we were, of course, unable to penetrate to the spectral region where the peptide chromophore absorbs. Consequently, we carried out a study in hexafluoro-2-propanol and found absorption bands and Cotton effects attributable to side-chain order only. We have no evidence for helicity of the main chain. It is, of course, possible that aromatic transitions overlapping the peptide region obscure the bands associated with amides in the helical array. This is doubtful because the spectrum below 275 mμ is similar to that of nonhelical polypeptides. In trifluoroacetic acid we observe no Cotton effects because the *o*-hydroxyaromatic chromophore is protonated but side chains are unable to organize themselves into an ordered array. That an ordered array is essential for a Cotton effect can be seen by the fact that we observe proper ultraviolet bands for the model compound but absolutely no ellipticity.

Experimental Section

Commercial Materials and Solvents. L-*p*-Nitrophenylalanine was obtained from the Cyclo Chemical Corp. and was used without further purification. *p*-Cresol was purchased from the Aldrich Chemical Corp. and was distilled before use. Trifluoroacetic acid and hexafluoro-2-propanol were obtained from the Aldrich Chemical Corp. and were used without further purification. Tetrahydrofuran, used for polymerization, and hexane, (Matheson Coleman and Bell) were refluxed over and distilled from sodium. *N,N*-Dimethylacetamide, used for circular dichroism and absorption measurements, was obtained from Matheson Coleman and Bell (Spectroquality grade).

Apparatus and Measurements. Ultraviolet and visible spectra were obtained using a Cary Model 14 and/or a Perkin-Elmer Model 350 double-beam spectrophotometer thermostated at 25°. The measurements were carried out in 0.1-, 0.2-, or 1.00-mm cylindrical cells (Optical Cell Co. Brentwood, Md.). Circular dichroism data were obtained on a Cary Model 60 spectropolarimeter thermostated at 27° equipped with a circular dichroism attachment with 0.1-mm (with trifluoroacetic acid) and 1.00 mm (with dimethylacetamide and hexafluoro-2-propanol) cylindrical cells.

Preparation of Compounds. L-*p*-Aminophenylalanine, $N^{\alpha,\omega}$-dicarbobenzoxy-L-*p*-aminophenylalanine, and N^{ω}-carbobenxoxy-L-*p*-aminophenylalanine N^{α}-carboxyanhydride were all prepared according to the procedure reported recently in the literature (Goodman and Peggion, 1967).

L-*p*-(2′-Hydroxy-5′-methylphenylazo)phenylalanine. The diazonium salt of L-*p*-aminophenylalanine was prepared according to the procedure of Bar-Eli and Katchalski (1963). The α-amino acid (4.0 mmoles) in 1 N HCl (4 ml) was diazotized with 0.5 N sodium nitrite solution (6 ml) at 0°. The solution was then buffered with 2 N potassium bicarbonate (20 ml) and diluted with 10% sodium acetate solution (40 ml). Freshly distilled *p*-cresol (4.1 mmoles) in water was added in one portion to the vigorously stirred mixture. The yellow precipitate was filtered and washed with cold water, acetone, and methanol. The crude material was used in the following step without further purification, mp 280° dec. The final yield was 88%.

N-Acetyl-L-*p*-(2′-hydroxy-5′-methylphenylazo)-phenylalanine. The free α-amino acid (2 mmoles) was converted into the *N*-acetyl derivative with acetic anhydride in 2 N sodium bicarbonate solution at 0°. By addition to the reaction mixture of HCl a precipitate was obtained. It was filtered, washed with cold water, and dissolved in ethyl acetate, then washed with a saturated solution of NaCl. The organic solution was dried over $MgSO_4$. The solvent was removed *in vacuo* and the resulting yellow solid was dissolved again in $CHCl_3$ and reprecipitated with *n*-hexane. The yield in the final product (mp 130–140) was 82%.

Poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine. N^{ω}-Carbobenzoxy-L-*p*-aminophenylalanine N^{α}-carboxyanhydride (1.0 g) was dissolved in 50 ml of freshly distilled dry tetrahydrofuran. A solution of sodium methoxide in methanol (0.1 N $NaOCH_3$) was used as an initiator ($A/I = 100$). The polymerization was allowed to proceed at room temperature for 5 days. The extremely viscous reaction mixture was poured into vigorously stirred cold *n*-hexane. The polymer was reprecipitated and dried giving 0.7 g of white, very fibrous polymer.

Poly-L-*p*-aminophenylalanine. Poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine (500 mg) was dissolved in 30 ml of trifluoroacetic acid and dry HBr was bubbled through the clear solution at room temperature for about 30 min. The precipitate was washed with ether and dried *in vacuo;* yield, 270 mg of a yellowish powdered polymer (~70% yield).

Poly-L-*p*-(2′-hydroxy-5′-methylphenylazo)phenylalanine. Poly-L-*p*-aminophenylalanine (162 mg) was diazotized according to a procedure reported in an earlier paper (Goodman and Benedetti, 1968). The diazonium salt was coupled with freshly distilled *p*-cresol to yield the desired polyazoaromatic peptide. The reaction mixture was dialyzed continuously against water for 3 days. Subsequent lyophilization yielded 250 mg of a reddish-brown powder (88% yield).

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A Method for the Complete S Sulfonation of Cysteine Residues in Proteins*

William W.-C. Chan

ABSTRACT: A new method is described for the complete sulfonation of protein SH groups under mild conditions. The protein is treated with sodium sulfite and catalytic amounts of cysteine in the presence of oxygen and 8 M urea.

When applied to rabbit muscle aldolase, complete sulfonation was obtained within 1 hr. The reaction was shown to be specific for SH groups from studies of the extent of the reaction and the electrophoretic pattern of the product. S-Sulfonated aldolase was enzymatically inactive but after suitable treatment with β-mercaptoethanol was reconstituted to give the fully active enzyme. The 100% regeneration of enzyme activity suggests that the method might be suitable for studies where subsequent recovery of biological activity is desired. In contrast, the S-sulfonated aldolase prepared by two other methods gave little or no activity after similar treatment. The reaction requires the addition of cysteine which may be replaced by β-mercaptoethylamine but not by β-mercaptoethanol or dithiothreitol. Under the conditions studied complete sulfonation occurs in the pH range 7.0–8.5 but little reaction takes place at pH 9.5 or higher. These findings suggest a role for the protonated amino group of cysteine in the reaction mechanism. Lactate dehydrogenase and pepsinogen were also completely sulfonated by this method. It is therefore suggested that the method may be generally applicable to proteins containing cysteine or cystine residues.

Sulfitolysis has been frequently used for the cleavage of disulfide bonds in proteins (Cole, 1967). If the reaction is allowed to proceed in a dissociating medium (*e.g.*, at high concentrations of urea or guanidine hydrochloride) and in the presence of an oxidizing agent, all the half-cystine residues can be converted into the *S*-sulfonate cysteine derivative. The completely S-sulfonated proteins so obtained are useful in the separation of polypeptides since they are stable in neutral and acidic conditions (Swan, 1957). A distinct advantage of

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