bined effects of free-radical scavenging and metal chelation. Pretreatment with resin results in the stabilization of L-ascorbic acid solutions in phosphate buffer at pH 7.2 and decreases the loss of viscosity of hyaluronic acid, as shown in Figure 4. The amount of L-ascorbic acid present was determined by the 2,6-dichloroindophenol method.[12] In the presence of solid resin, a loss of L-ascorbic acid was barely detectable, and the rate of depolymerization was very slow.

Discussion

Depolymerization of hyaluronic acid and autoxidation of L-ascorbic acid proceed only slowly in solutions that have been rigorously purified by sequestering resin (Chelex 100), and the reaction in untreated solutions is thus dependent on metal catalysis brought about by the impurities that are present in commercial phosphate buffer and in hyaluronic acid solutions. Thus, at 0.2 *M*, commercial phosphate salts contain up to 5 μM iron; in addition, hyaluronic acid binds iron, a property used in the histochemical localization of mucopolysaccharides.[13]

When Chelex 100 is kept in contact with, rather than separated from, the reaction milieu, the rate of autoxidation of L-ascorbic acid and of the concomitant depolymerization of hyaluronic acid is drastically reduced. This effect may possibly arise by prevention of an accumulation of metal impurities that originate either from the atmosphere or that are leached from the glassware by the phosphate buffer.

The rates of depolymerization of hyaluronic acid, as measured by specific fluidity, are directly proportional to the quantity of catalyst for the first 10 min of the reaction period.[5] A similar relationship was observed by Humphreys and Howells[14] for the degradation of alginate by γ-irradiation. The level of copper ions required for measurable increase in the rate of depolymerization of hyaluronic acid (about 0.25 μM) represents 16 ppb of copper ions in water compared to 59 ppm for L-ascorbic acid (0.33 m*M*).

The involvement of metal catalysts in these autoxidative reactions is in agreement with studies on the degradation of citrate by L-ascorbate in Krebs–Ringer phosphate.[15] However, in that study, EDTA was observed to inhibit the ascorbate-induced degradation of citric acid, whereas its presence was shown to aid catalysis in this investigation. The anomaly may perhaps be explained by alternative reliance on iron catalysis, which will be aided by EDTA, as opposed to copper catalysis, which will be inhibited by EDTA.[5] The activity of the iron–EDTA complex in this system for extensively depolymerizing simple and complex carbohydrates has recently been indicated.[16]

It is clear that most if not all previous studies of the oxidation effects of L-ascorbic acid have involved metallic catalysis, presumably by iron or copper ions. The function of the ascorbic acid in such systems is to regenerate the ferrous or cuprous ions which are the actual catalysts. Presumably, this action takes place in biological systems,[17,18] but, since other biological substances and other isomers of L-ascorbic acid are active *in vitro* but not *in vivo*, some sites of action would appear to involve stereoisomeric components such as enzymes.

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(13) C. W. Hale, *Nature (London)*, **157**, 302 (1946).
(14) E. R. Humphreys and G. R. Howells, *Carbohyd. Res.*, **16**, 65 (1971).
(15) M. P. Lamden and A. S. Kunin, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 11, 328 (1970).
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Communications to the Editor

A Laser Raman Spectroscopic Study of the Effect of Solvation on the Conformation of Ribonuclease A

Sir:

Lyophilization, freeze drying, is a common method of reducing protein solution to dryness. Most proteins are not denatured by this process and may be stored in the dry state for long periods of time without deterioration of the sample. It is believed tht part of the water is still tightly bonded to protein molecules in the powder and cannot be released even on drastic drying.[1] The major change in this process is the removal of unbonded and loosely bonded water molecules from the frozen sample and the replacement of a certain number of protein–solvent contacts by protein–protein contacts. However, it is not known whether the removal of water from part of the molecular surface may have an effect on the conformation of a protein. In this communication, we wish to present the Raman spectra of a model protein, ribonuclease A, in the powder form and in aqueous solution. These spectra will be compared quantitatively and the spectral differences will be interpreted in terms of main chain and side chain conformational changes.

The Raman spectra of RNase A in solution were first obtained and interpreted by Lord and Yu[2] in 1970. The powder spectra were not reported at that time because of the experimental difficulties associated with high background scattering. Recently, Koenig[3] obtained a preliminary Raman spectrum of the same protein in the solid state and compared it to that in solution. He noted that the Raman lines of the aqueous solution spectra are sharper than the lines of the solid spectra. In Figure 1 we present our higher resolution Raman spectra of RNase A in the powder form and in

(1) E. Ellenbogen, *J. Amer. Chem. Soc.*, 77, 6634 (1955).
(2) R. C. Lord and N-T. Yu, *J. Mol. Biol.*, **51**, 203 (1970).
(3) J. L. Koenig, *J. Polym. Sci., Part D*, in press.

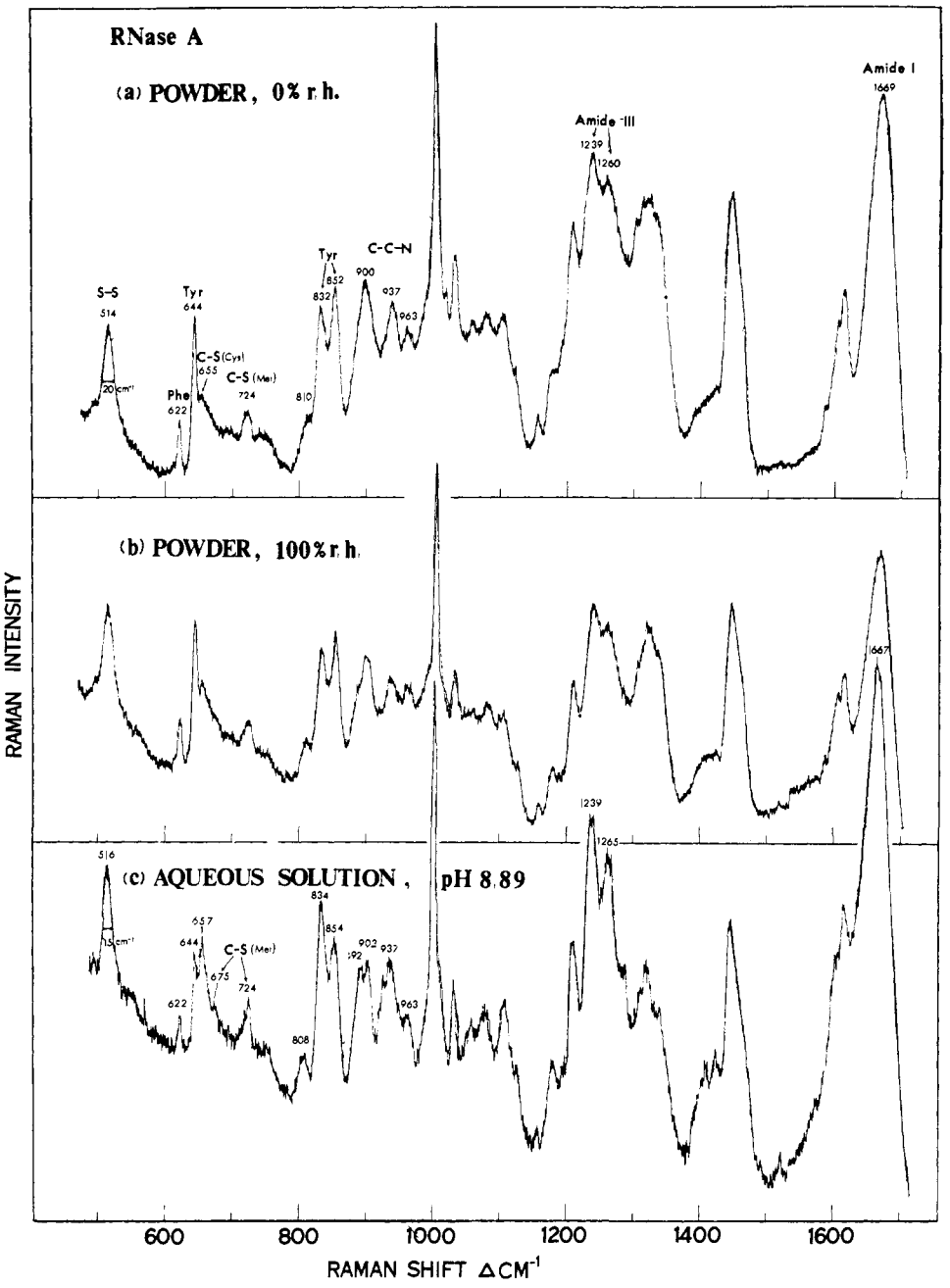

Figure 1. Raman spectra of ribonuclease A in the solid and aqueous solution. (a) Spectrum of the lyophilized powder of RNase A in 0% relative humidity (rh): slit width ($\Delta\sigma$), 4 cm^{-1} (200 μ); sensitivity (s), 5000 counts per second (cps) full scale; rate of scan (γ), 10 cm^{-1}/min; standard deviation (sd), 0.7%; laser power (p) at the sample, 153 mW at 514.5 nm. The sample in powder form was packed into a conical depression at the end of an $^1/_8$-in. gold-plated copper rod, which was fastened in a Thermovac flask equipped with an "O" ring and a vacuum tight stopcock. The powder was dried *in vacuo* at 25° over phosphorus pentoxide for 2 hr before the experiment. The flask containing the sample and phosphorus pentoxide was then filled with dry nitrogen at 1 atm and used for laser Raman scattering work. (b) Spectrum of the lyophilized powder of RNase A in 100% rh $\Delta\sigma$, 4 cm^{-1} (200 μ); s, 5000 cps; γ, 10 cm^{-1}/min; sd, 0.5%; p, 160 mW. The powder was equilibrated at room temperature with saturated H_2O vapor pressure (about 24.00 mm) for 5 hr. (c) Spectrum of RNase A in aqueous solution at pH 8.89: $\Delta\sigma$, 4 cm^{-1} (200 μ); s, 5000 cps; γ, 25 cm^{-1}/min; sd, 1%; p, 200 mW. The solution sample at 200 mg/ml concentration and pH 8.89 was obtained by dissolving the required amount of the powder used in a and b without adding HCl or NaOH solution.

solution. Here two powder spectra are reported with one taken in 0% and the other in 100% relative humidity at 25°. No noticeable spectral differences were observed between the two spectra. On comparing the powder and solution spectra, however, several regions have revealed striking changes.

Particularly interesting spectral changes involve the lines at 644, 832, and 852 cm^{-1} due to the ring vibrations of tyrosine residues.[2] Upon dissolution, the intensities of the lines at 644 and 852 cm^{-1} have decreased considerably (relative to the line at 832 cm^{-1}). Since three of the six tyrosines (Tyr-73, -76, and -115) have a normal pK_a value[4,5] (10–10.2) and are presumably accessible to the water molecules,[6] one might interpret

(4) D. Shugar, *Biochem. J.*, **52**, 142 (1952).
(5) R. W. Woody, M. E. Friedman, and H. A. Scheraga, *Biochemistry*, **5**, 2034 (1966).
(6) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3984 (1967).

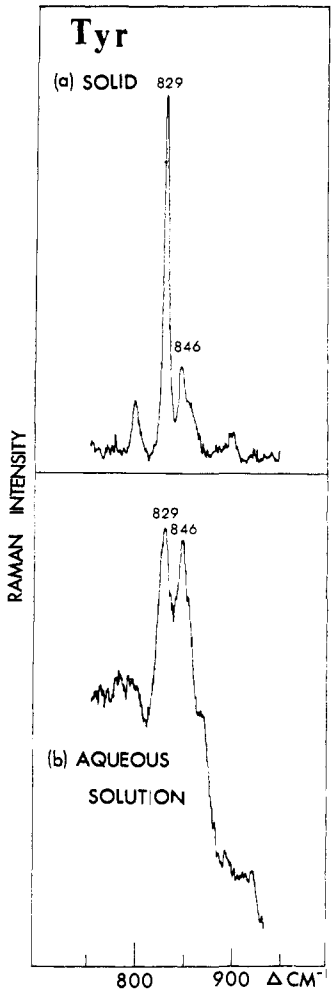

Figure 2. Raman spectra of tyrosine in the solid and aqueous solution. The concentration of solution was 0.1 *M* and the pH value 1.0.

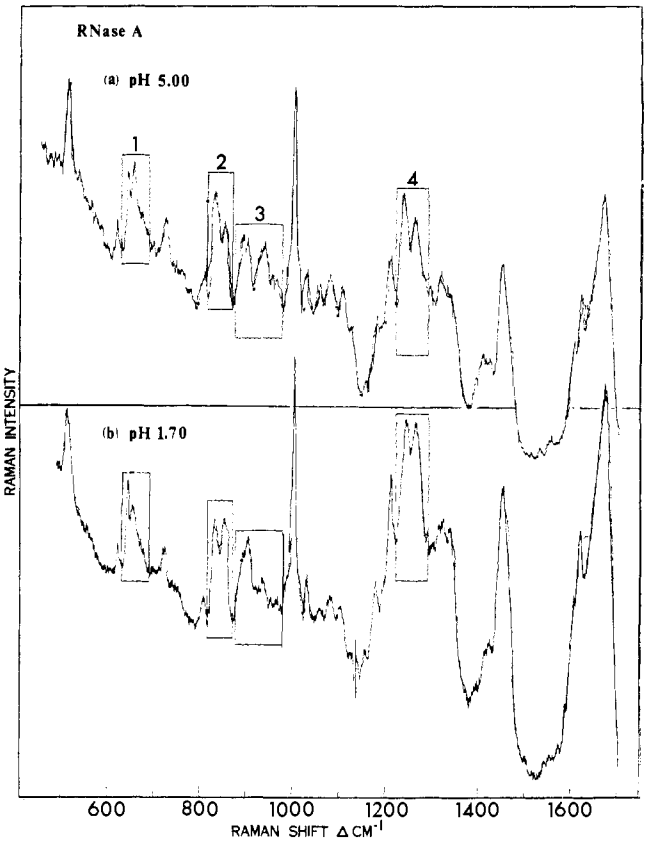

Figure 3. (a) Raman spectrum of aqueous RNase A at pH 5.00: $\Delta\sigma$, 4 cm^{-1} (200 μ); *s*, 2500 cps; γ, 25 cm^{-1}/min; sd, 1%; *p*, 120 mW. (b) Raman spectrum of aqueous RNase A at pH 1.70: $\Delta\sigma$, 4 cm^{-1} (200 μ); *s*, 2500 cps; γ, 10 cm^{-1}/min; sd, 0.5%; *p*, 130 mW (450–1130-cm^{-1} region), 150 mW (1130–1700-cm^{-1} region). The concentration for a and b was 200 mg/ml.

these intensity changes as due to the solvation of these exposed tyrosines when the protein molecule passed into the solution. However, this is not an acceptable explanation. Two lines of evidence strongly suggest that the observed changes in intensity may be attributed to the change in the local environment of the other three tyrosines (*i.e.*, Tyr-25, -92, and -97) having an abnormally high pK_a value[4,5] (greater than 11) and that this environmental change probably is the result of conformation changes in the vicinity of these groups. The first line of evidence comes from the spectra of solid and aqueous tyrosine. As one can see from Figure 2, the direction of the relative intensity change near 840 cm^{-1} upon dissolution is opposite to that of RNase A (Figure 1). The second line of evidence involves the spectral differences between the pH 5.00 and 1.70 spectra (Figure 3). There are no significant differences between the spectra of pH 8.89 and 5.00. The Raman lines at 644 and 852 cm^{-1} have increased their intensities relative to the 832-cm^{-1} line, suggesting a further change in the local environment of the three "buried" tyrosines. This time the environmental change may be caused not only by the disruption of tyrosyl–carboxylate ion interactions[7] through protonation but also by the partial unfolding of the main chain as evident from the spectral change in the amide III region. Both effects combined may bring the tyrosyl groups from a hydrophobic region to an aqueous one and give rise to the expected intensity increase of the lines at 644 and 852 cm^{-1}. If the above interpretation is correct, one may conclude that the intensity of the ring vibrations of tyrosine is sensitive to changes in conformation. It should be mentioned here that in the earlier studies of lysozyme,[2] ribonuclease,[8] α-chymotrypsin,[8] and insulin[9,10] the aromatic ring vibrations were thought to be independent of protein conformation both in frequency and intensity.

Small but definite spectral changes occur in the S–S and C–S stretching vibrations region (500–730 cm^{-1}). According to Lord and Yu,[2] the lines at 514 and 655 cm^{-1} are assigned to the S–S and C–S stretching of the four disulfide bridges (residues 26–84, 40–95, 58–110, and 65–72). In the powder spectra (Figure 1a and b), the half-width of the 514-cm^{-1} line is 20 cm^{-1} and the intensity ratio of the C–S to S–S lines is about 0.53. In the solution spectrum (Figure 1c) the half-width of the S–S line is only 15 cm^{-1} and the intensity ratio is somewhat greater, *i.e.*, about 0.91. Since the line width is a reflection of the uniformity of the disulfide

(7) Woody, *et al.*,[5] presented evidence that the three "buried" tyrosyl residues in native RNase A in solution were involved in tyrosyl–carboxylate ion hydrogen bonding interactions, but the X-ray crystal structure of Wyckoff, *et al.*,[6] shows that Tyr-92 may be hydrogen bonded to the backbone at Asp-38, the hydroxyl of Try-25 is near the carboxyl of Asp-14, and Tyr-97 is probably hydrogen bonded to the backbone at Lys-41.

(8) R. C. Lord and N-T. Yu, *J. Mol. Biol.*, **50**, 509 (1970).
(9) N-T. Yu and C. S. Liu, *J. Amer. Chem. Soc.*, **94**, 3250 (1972).
(10) N-T. Yu, C. S. Liu, and D. C. O'Shea, *J. Mol. Biol.*, in press.

geometry and the intensity ratio a measure of the C–S–S bond angles,[2,8] the observed spectral changes mean that the geometry of the disulfide linkages is more uniform in solution than in the solid state and that the C–S–S bond angles are smaller in solution.[2,8] The line at 724 cm^{-1} was assigned to the C–S stretching of *trans*-methionines by Lord and Yu.[2] When a comparison is made between the powder and solution spectra, a slight sharpening of the 724-cm^{-1} line is noticeable.

In the amide III region (1220–1300 cm^{-1}), two resolved lines were observed at 1239 and 1260 cm^{-1} in the powder spectra. According to a recent study on the Raman spectra of glucagon in various conformational states, Yu and Liu[11] concluded that the α helical, random coil, and β structure of a protein should have the amide III vibrational frequencies at 1266, 1248, and 1232 cm^{-1}, respectively. On the other hand, the X-ray diffraction studies of RNase A[12] and RNase S[6,13] have revealed that the main chain of the molecule may roughly be divided into two parts with three sections of α helix (2–12, 26–33, and 50–58, totaling approximately 15% of the molecule) in the first half, and the bulk of the β structure (somewhat irregular) in the second half. With this information at hand, we assigned the line at 1239 cm^{-1} to the β structure and the one at 1260 cm^{-1} to the α helices. Upon dissolution, the 1260-cm^{-1} line shifted to 1265 cm^{-1} and both lines sharpened.

Other manifestations of the conformational changes occur near 810 and between 870 and 980 cm^{-1}.

It is interesting to note that although the molecule consists of two distinct structural components in the main chain, the amide I region does not show this. Since the α helix and β structure are expected to have the amide I frequencies near 1660 and 1672 cm^{-1}, respectively,[11] the observed single line at 1669 cm^{-1} in the powder spectra (or 1667 cm^{-1} in the solution spectra) is probably the superposition of these two lines.

At the present time we are investigating the Raman spectra of RNase A crystals to determine whether the structure of the enzyme in crystals is the same as in solution or resembles that in the lyophilized powder. We are also investigating the S-peptide–S-protein binding interaction of RNase S both in crystals and in solution. Results on this aspect of the work will be presented in a future publication.

Acknowledgments. This work was supported by the U. S. National Institute of General Medical Sciences (GM-18894-01) and by the Research Corporation (a Frederick Gardner Cottrell Grant).

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(12) G. Kartha, J. Bello, and D. Harker, *Nature (London)*, **213**, 862 (1967).
(13) H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, and F. M. Richards, *J. Biol. Chem.*, **245**, 305 (1970).

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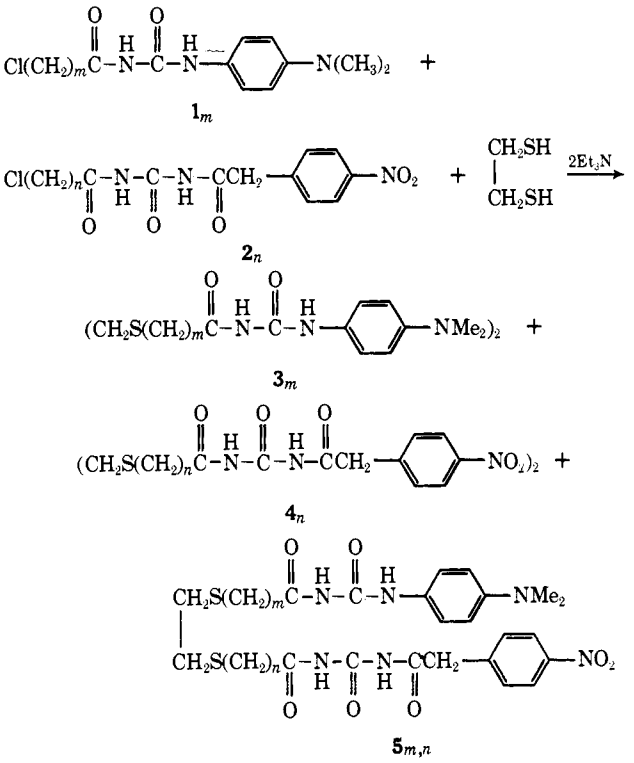
Selective Formation of Symmetrical Bissulfides

Sir:

We report a remarkable selectivity in the dialkylation of ethanedithiol with alkyl halides of general formulas **1** and **2**. The model halides[1] were easily prepared in high yields by treatment of acyl isocyanates[2] with *N,N*-dimethyl-*p*-phenylenediamine in acetonitrile at room temperature or with *p*-nitrophenylacetamide in boiling dioxane.



1$_m$ + **2**$_n$ $\xrightarrow{2Et_3N}$ **3**$_m$ + **4**$_n$ + **5**$_{m,n}$

In a typical experiment, a solution of ethanedithiol (10 mol) and triethylamine (22 mmol) in 50 ml of acetonitrile was added to a suspension of **1**$_1$ (10 mmol), mp 195–196°, and **2**$_2$ (10 mmol), mp 164.5–165.0°, in 200 ml of acetonitrile with stirring at room temperature over a period of 2 hr. Stirring was continued for an additional 12 hr at room temperature. The reaction mixture was evaporated, treated with water, and dried *in vacuo*. The resulting brown solid gave **3**$_1$ (38%), mp 236.0–236.5° dec (DMF–acetonitrile) [*Anal.* Calcd for $C_{24}H_{32}N_6O_4S_2$: C, 54.13; H, 6.06; N, 15.78; S, 12.02. Found: C, 54.11; H, 5.89; N, 15.95; S, 12.16], and **4**$_2$ (31%), mp 194–195° dec (acetonitrile) [*Anal.* Calcd for $C_{26}H_{28}N_6O_{10}S_2$: C, 48.15; H, 4.35; N, 12.96; S, 9.87. Found: C, 48.07; H, 4.24; N, 12.83; S, 10.16], on successive treatments with boiling DMF–acetonitrile, acetonitrile at room temperature, and boiling acetonitrile. No unsymmetrical bissulfide (**5**$_{1,2}$) could be isolated from the residual solid. All the results are summarized in Table I. The structural assignment of the bissulfides is based on their elemental analyses and the infrared and nmr spectra. The infrared spectra of **3**$_1$, **4**$_1$ (mp 172–175° dec), and **5**$_{1,1}$[3] [*Anal.* Calcd for $C_{24}H_{28}N_6O_7S_2$: C, 50.00; H, 4.90; N, 14.58; S, 11.11. Found: C, 49.81; H, 4.73; N, 14.85; S, 11.02] were readily distinguishable. The structures of bissulfides were further confirmed by

(1) All new compounds in this paper gave satisfactory elemental analytical values.
(2) A. J. Speziale and L. R. Smith, *J. Org. Chem.*, **27**, 3742 (1962).
(3) The DTA curve of **5**$_{1,1}$, which was recrystallized from dichloroethane, under a nitrogen atmosphere, showed two endothermic peaks at 126 and 175°, suggesting that **5**$_{1,1}$ might be a liquid crystal. In addition, this compound is yellow in a solvent and turned pale orange in air. This change in color is reversible.