The Optical Activity of the Disulfide Bond in L-Cystine and Some Derivatives of L-Cystine[1]

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Abstract: Spectral propertie of L-alanine, L-cysteine, L-cystine, N,N′-diacetyl-L-cystine (DAC), N,N′-diacetyl-L-cystine bismethylamide (DACMA), N-acetyl-L-cysteine methylamide (reduced DACMA), and oxidized and reduced glutathione (GSSG and GSH) were measured. The optical rotatory dispersion (ORD) curves of alanine and cysteine in the region above 190 mμ are dominated by a positive Cotton effect associated with the $n \rightarrow \pi^-$ carboxyl transition. With cystine, the observed curve is resolved by means of a computer analysis into a set of three Cotton effects which are consistent with the data obtained from measurements of the circular dichroism (CD) and ultraviolet (uv) spectra. The rotational strengths and positions of these Cotton effects at low pH are: -1.69×10^{-40} erg cm³ (252 mμ), $+19.9 \times 10^{-40}$ erg cm³ (218 mμ), and -75.0×10^{-40} erg cm³ (187 mμ). Although the disulfide optical activity is somewhat modified in the case of the cystine derivatives, the general characteristics are similar to those of cystine. A computer analysis of the ORD of DACMA yielded a set of disulfide Cotton effect parameters which are used to calculate the ORD and CD curves characteristic of the disulfide group. The rotational strengths and positions of the disulfide Cotton effects of DACMA are: -1.64×10^{-40} erg cm³ (262 mμ) and -33.7×10^{-40} erg cm³ (199 mμ). The molar rotation of the disulfide group at 589 mμ is −436°. At 210 mμ, the position of the deep disulfide trough, the molar rotation is approximately −24,000°. The significance of the disulfide contributions to the ORD of proteins will depend on the magnitude of the contributions from all other transitions. It is calculated that disulfide Cotton effects would account for more than 10% of the observed rotations in the region near 210 mμ for a polypeptide containing two S–S bonds per hundred residues when the helix content is between 25 and 40%. Differences in the rotational strengths of the two S–S transitions as well as differences in their sensitivity to modification of other groups of the molecule suggest that the 199-mμ Cotton effect may be directly related to the inherent asymmetry of the disulfide group, whereas the 262-mμ Cotton effect may result primarily from environmental perturbations. A possible explanation is offered for this difference in optical activity based on the assignments for these transitions.

It has long been known that cystine shows unusual optical rotatory properties, relative to other natural amino acids,[2] which have been attributed to an inherent asymmetry of the disulfide bond.[3] Since optical rotatory dispersion (ORD) is often used in investigations of the conformation of proteins in solution and to detect conformational changes, it is essential to determine the nature and significance of any contribution associated with disulfide bonds to the ORD of the proteins containing them. This is of particular importance in investigations of the changes in the ORD of proteins and peptides when constituent disulfide bonds are cleaved. Such changes may be the result of conformational changes in the protein, particularly in the secondary structure, or, alternatively, may be due to the loss of the disulfide contribution to the rotation. There has been insufficient evidence to permit evaluation of the significance of the latter alternative.

In this communication, we present the results of investigations of spectral and rotatory properties of L-alanine, L-cysteine, L-cystine, and several derivatives of cystine which contain secondary amide bonds. These results, obtained for the most part in the spectral region extending from 300 mμ to approximately 190 mμ, indicate that the ORD of all three amino acids includes a positive Cotton effect associated with the $n \rightarrow \pi^-$ transition of the carboxyl group. In addition, there is a significant negative contribution to the far ultraviolet ORD of L-cysteine which appears to be related to one or more transitions of the sulfhydryl group located below 190 mμ. With cystine, two negative Cotton effects together with the carboxyl Cotton effect are found to account for the observed ORD. These negative Cotton effects, located at 252 and 187 mμ at pH 0.35, are associated with transitions of the disulfide bond. The results obtained for the cystine derivatives indicate that the rotations from the disulfide Cotton effects constitute the major part of the observed ORD of these compounds. In another communication,[4] related studies on polypeptides and heterodetic cyclic peptides containing disulfide bonds have been reported.

Experimental Section

Materials. L-Alanine, L-cystine, and N,N′-diacetyl-L-cystine monohydrate were purchased from the California Corporation for Biochemical Research, and were reported to be chromatographically homogeneous. L-Cystine, oxidized glutathione, and reduced glutathione were purchased from the Mann Research Laboratories, Inc. The cystine and oxidized glutathione were chromatographically homogeneous, while the reduced glutathione showed only a trace amount of oxidized glutathione. L-Cysteine was purchased from the Nutritional Biochemicals Corp. ($[\alpha]^{20}D$ +6.5° (*c* 2, 5 *N* HCl)). N-Ethyl-5-phenylisoxazolium-3′-sulfonate was purchased from Pilot Chemical Co. Acetonitrile (spectrograde), triethylamine, methylamine hydrochloride, and β-mercaptoethanol (all reagent grade) were purchased from Eastman Organic Chemicals Co.

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(2) J. H. Van't Hoff, "Die Lagerung der Atome im Raum," 2nd ed, Springer-Verlag, 1894, as referred to by L. F. Fieser, *Rec. Trav. Chim.*, 69, 410 (1950).

(3) J. Strem, Y. Krishna-Prasad, and J. Schellman, *Tetrahedron*, 13, 176 (1961).

(4) D. L. Coleman and E. R. Blout in "Conformations of Biopolymers," Vol. 1, G. N. Ramachandran, Ed., Academic Press Inc., London, England, 1967, p 123.

β-Mercaptoethanol was distilled under nitrogen (bp 49° (13 mm)) and stored in the dark under nitrogen at 4°.

2,2′-Dithiodiethanol was formed by air oxidation of an alkaline solution of mercaptoethanol in the presence of catalytic amounts of manganese sulfate for 36 hr.[5] The solution was extracted with ether which was then removed by flash evaporation, leaving a residual oil which solidified at −30°. The solid was taken up in warm ether, and the product was crystallized from the ether solution at 4°. After recrystallization from ether, the melting point was 27.5° (capillary and electrically heated block, uncorrected).

Anal. Calcd for $C_4H_{10}O_2S_2$: C, 31.1; H, 7.0; S, 41.5. Found: C, 31.4; H, 6.6; S, 41.8.

N,N′-Diacetyl-L-cystine bismethylamide was synthesized using an isoxazolium salt[6] as the condensing agent. N,N′-Diacetyl-L-cystine monohydrate (1.0 g, 2.92 mmol), dried for 2.5 hr over P_2O_5 *in vacuo*, was added to N-ethyl-5-phenylisoxazolium-3′-sulfonate (1.48 g, 5.84 mmol), and acetonitrile (20 ml) was added to the dry mixture. The suspension was cooled to 4°, and triethylamine (2.0 ml of a 41% solution (v:v) of triethylamine in acetonitrile, 5.84 mmol of triethylamine) was added slowly with stirring. After stirring for 3 hr at 4°, methylamine hydrochloride (0.39 g, 5.84 mmol), dried 2 hr at 120° over P_2O_5 *in vacuo*, was added, followed by triethylamine (2.0 ml of 41% solution in acetonitrile). After stirring at 4° for 22 hr, the flask was warmed to room temperature and stirring continued for an additional 44 hr. The reaction mixture, which contained a fine suspension of solid material, was centrifuged and the supernatant decanted. The precipitate was resuspended in acetonitrile and centrifuged. After decanting the supernatant, the precipitate was taken up in hot absolute ethanol (150 ml) and crystallized at 4°. The fine white solid was filtered, washed with cold absolute ethanol, and dried, yield 0.31 g (30%), mp 261° dec (Köfler block).

Anal. Calcd for $C_{12}H_{22}N_4O_4S_2$: C, 41.1; H, 6.3; N, 16.0; S, 18.3. Found: C, 41.1; H, 6.1; N, 15.9; S, 18.2.

Methods. Electrolytic reduction of the disulfide bond of N,N′-diacetyl-L-cystine bismethylamide was carried out using an electrolytic desalter, Model B-1930, obtained from the Research Specialties Co., Berkeley, Calif., according to the procedure described by Benesch and Benesch.[7] Reductions were carried out with potentials of 10–15 V (currents 0.04–0.10 A) for periods of 15–30 min. Extent of reduction was determined by titrating the sulfhydryl groups with *p*-chloromercuribenzoate by Boyer's method.[8]

pH measurements were made with a Radiometer pH meter, Model pHM4c. Solutions used for measurement of pH were not used for measurement of the far-ultraviolet spectrum since they were found to be contaminated with small amounts of KCl from the calomel electrode.

Absorption spectra were measured with a Cary Model 15 recording spectrophotometer equipped with special phototubes permitting measurements to be made to 175 mμ. Scans to 190 mμ were made without purging with nitrogen. Measurements made from 195 to 180 mμ were made after purging the instrument with dry nitrogen. Since opening the cell compartments disturbs the nitrogen atmosphere in that part of the instrument, it was necessary after changing the cells to reflush the cell compartments before making further measurements. During the course of flushing the cell compartments, repeated scans through the wavelength region of interest were made until they showed no further change with time, indicating that the atmosphere of the cell compartment had reached a steady state. Measurements made in the region between 190 and 195 mμ with and without purging gave the same results.

Optical rotatory dispersion measurements were made with a Model 60 Cary recording spectropolarimeter. Cells, selected for minimal birefringence, had path lengths which ranged from 0.500 to 0.00099 dm. The positions of these cells in the cell holder could be reproduced sufficiently accurately that differences in the observed rotations associated with differences in the positioning of the cell were not significant. Measurements made over different wavelength regions overlapped extensively to check for reproducibility. Since substantial artifacts have been observed in a spectropolarimeter of different make for solutions having high optical densities,[9] tests were carried out with the Cary 60 using potassium dichromate solutions. No rotational artifacts were observed with samples having optical densities as high as 2.0. Also the calibration of the instrument was checked using a solution of sucrose according to the method described by Samejima and Yang.[10] The results revealed excellent agreement in the far ultraviolet with the calculated rotations for sucrose.

Molar rotations were calculated using the equation

$$[\phi]_\lambda = \frac{\alpha_\lambda M}{cl}$$

where α_λ is the observed rotation (degrees) at wavelength λ, M is the molecular weight of the compound, c is the concentration in grams/100 ml, and l is the cell path length in decimeters.

Circular dichroism (CD) measurements were made with a modified Jouan dichrographe.[11] For amides and peptides, measurements could be made to approximately 220 mμ. With N-acetyl-L-cysteine methylamide and N,N′-diacetyl-L-cystine bismethylamide measurements were also made with a JASCO UV/ORD-5 optical rotatory dispersion recorder equipped with a circular dichroism attachment. With L-cystine at low pH, some measurements were made with the instrument built and described by Holzwarth.[12] At the time these measurements were made, a 0.070-in. electrooptic plate had been installed in this instrument, and the measurements could be made to nearly 200 mμ.

The circular dichroism, $(\epsilon_L - \epsilon_R)_\lambda$, is calculated using the relationship

$$(\epsilon_L - \epsilon_R)_\lambda = \frac{(K_L - K_R)_\lambda}{c'l'}$$

where $(K_L - K_R)_\lambda$ is the observed difference in absorption between left and right circularly polarized light, c' is the molar concentration, and l' is the path length in centimeters.

Although circular dichroism measurements were made, all results have been expressed in terms of the molar ellipticity, $[\theta]_\lambda$, which has the same units as $[\phi]_\lambda$, using the relationship

$$[\theta]_\lambda \approx 2.303\frac{4500}{\pi}(\epsilon_L - \epsilon_R)_\lambda$$

Analysis of ORD Curves.[13] The Cotton effect associated with an optically active electronic transition is related to the corresponding CD band by the Kronig–Kramers transform. If it is assumed that the CD band is Gaussian having the form

$$[\theta]_\lambda = [\theta^0]\exp(-[(\lambda - \lambda^0)/\Delta^0]^2) \qquad (1)$$

where $[\theta^0]$ is the value of $[\theta]$ at the extremum of the band, λ^0 is the position of the extremum, and Δ^0 is the half-width of the band, *i.e.*, the interval between λ^0 and the wavelength where $[\theta] = [\theta^0]e^{-1}$, then the value of $[\phi]_\lambda$ can be expressed in terms of these parameters according to eq 2[14]

$$[\phi]_\lambda \approx \frac{A\lambda^0}{\Delta^0}\left\{\exp\left[-\left(\frac{\lambda - \lambda^0}{\Delta^0}\right)^2\right]\int_0^{(\lambda-\lambda^0)/\Delta^0}\exp(y^2)dy - \frac{\Delta^0}{2(\lambda + \lambda^0)}\right\} \qquad (2)$$

where $A \equiv 2[\theta^0]\Delta^0/\pi^{1/2}\lambda^0$. When several optically active transitions are present, the observed rotation is the sum of the rotations calculated for the individual Cotton effects.

Nonlinear analyses of ORD curves were carried out on an IBM 1620 computer using a program written and described by Carver, *et al.*[14,15] The program calculates the total rotations for a set of Cotton effects over the wavelength range of interest, and adjusts the three parameters, A_i, λ_i^0, and Δ_i^0, for each Cotton effect to give the best weighted fit of the observed ORD curve. Weights are used to allow for the fact that the errors in the observed rotations vary with wavelength, generally becoming much greater in the far-ultra-

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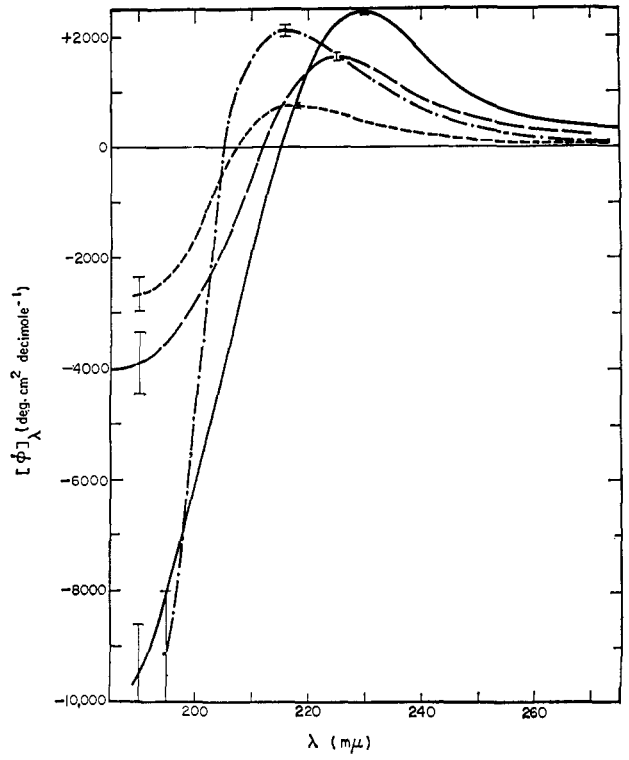

Figure 1. ORD of L-alanine (— —, pH 0.65; - - -, pH 6.5) and L-cysteine (——, pH 0.41; -·-·-, pH 5.2).

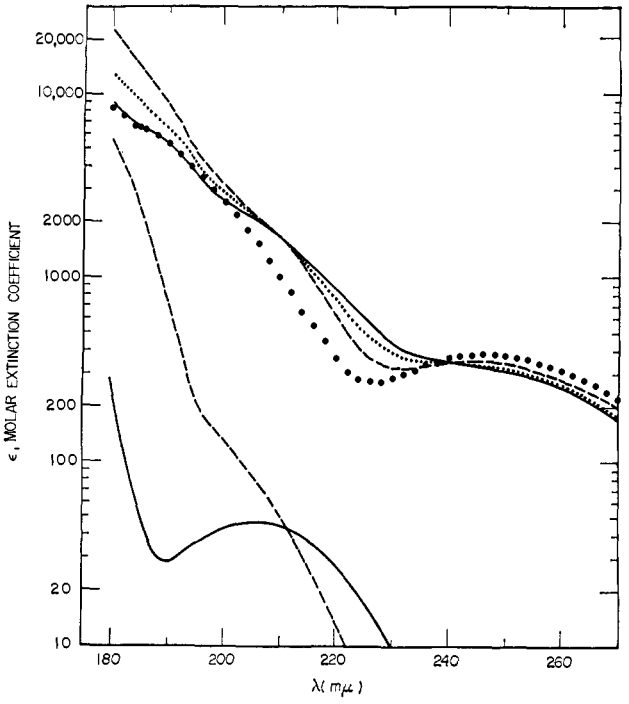

Figure 2. Absorption spectrum of L-cystine (——, pH 0.35; ·····, pH 1.63; - - -, pH 6.3), L-alanine (——, pH 0.65; - - -, pH 6.5), and 2,2′-dithiodiethanol (●, pH 6.5).

violet region of the spectrum. The weighted residual mean square (RESMS) is a measure of the fit attained. The method of assigning weights is that used by Carver.[15] The experimental point having the largest standard deviation is given a weight of unity, and the experimental RESMS is equal to the square of the standard deviation of that point. If the calculated curve fits the observed curve within experimental error, the calculated RESMS will be less than or equal to the experimental RESMS.

For estimating the values of A to be used as initial parameter values in the computer analysis, the following equations are useful: (a) from an isolated CD band

$$A \approx 1.132[\theta^0]\Delta^0/\lambda^0 \quad (3)$$

(b) from an isolated Cotton effect

$$A \approx 1.85[\phi]_{ext}\Delta^0/\lambda^0 \quad (4)$$

where $[\phi]_{ext}$ is the magnitude of the rotation at either extremum, λ^0 is taken as the position of the middle inflection point in the Cotton effect curve, and Δ^0 is approximately equal to the interval, $|\lambda_{ext} - \lambda^0|$. The rotational strength of the transition is related to the constant, A, by the equation

$$R \approx 1.09 \times 10^{-42}A \quad (5)$$

Results and Discussions

A. L-Alanine. Since alanine does not have a side group with transitions in the accessible region of the spectrum (above 185 mμ), its ORD should reflect the general properties of the contributions by the carboxyl and amino groups to the ORD of amino acids. As shown in Figure 1, there is a small positive Cotton effect in the ORD curve of alanine, at low pH, centered near 205 mμ. At neutral pH, this Cotton effect is shifted to near 199 mμ and is reduced in magnitude.

The absorption band associated with this Cotton effect is seen in Figure 2 for alanine at low pH. At neutral pH, the absorption spectrum has no discreet bands in the region investigated. However, a shoulder is observed near 200 mμ which suggests that the weak absorption band observed at low pH has been shifted to shorter wavelengths and is now hidden under the increased end absorption, an interpretation which is consistent with the observed blue shift of the alanine Cotton effect. The absorption band observed with alanine at low pH has also been observed with other amino acids[16] as well as with simple carboxylic acids[17,18] and has been assigned to the n → π^- transition of the carboxyl group.[18] Thus, the present results confirm previous suggestions[19,20] that the alanine Cotton effect could be associated with a transition of the carboxyl group. In addition, the effect of the ionization of the carboxyl group of alanine on its optical activity is characterized by both a shift in the position of the n → π^- Cotton effect to shorter wavelengths and a decrease in its rotational strength. Urry, *et al.*,[21] have interpreted the shift in position of the n → π^- carboxyl transition as arising from an increase in the energy of the π^- state which would be expected to accompany an increased stability of the π^+ state of the ionized form of the carboxyl group relative to that for the un-ionized form. Schellman[22] noted the decrease in $[\alpha]_D$ for alanine relative to the value at low pH and attributed it to a reduction in the rotational strength of a carboxyl transition due to the introduction of a plane of symmetry into the group upon ionization. The results shown in Figure 1 substantiate this explanation, although it is clear that the shift in position of the Cotton effect with pH also influences the value of $[\alpha]_D$.

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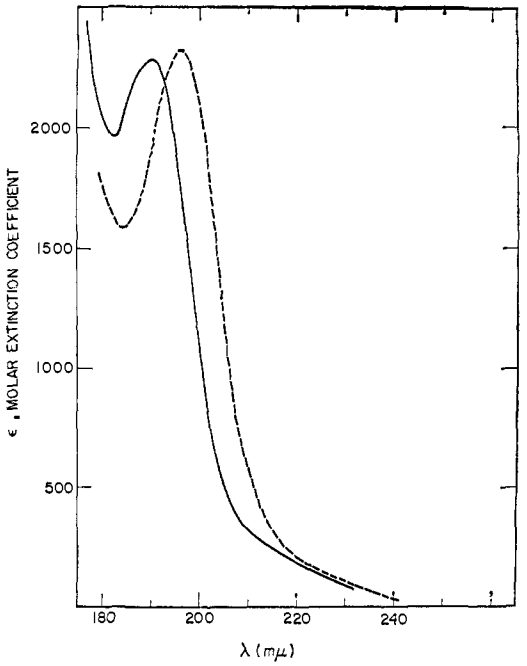

Figure 3. Absorption spectrum of L-cysteine (——, pH 0.41) and β-mercaptoethanol (– – –, pH 0.33).

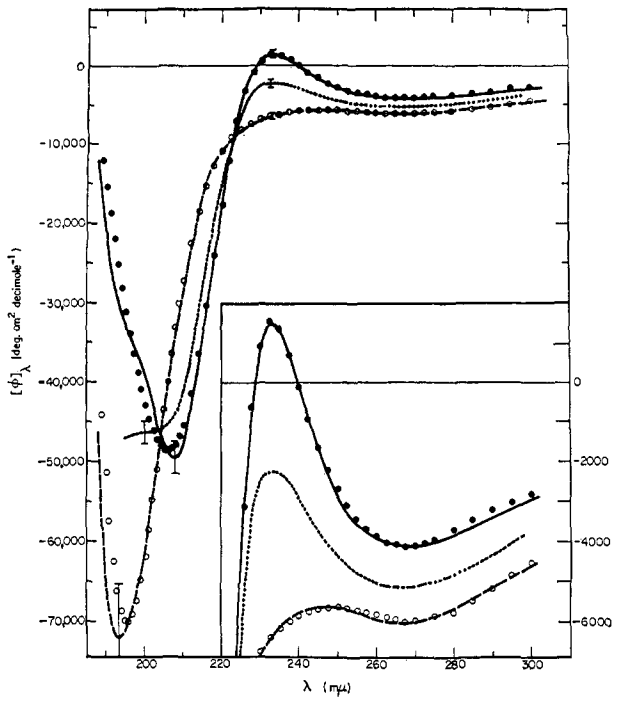

Figure 4. ORD of L-cystine (——, pH 0.35; · · · · ·, pH 1.63; – – –, pH 6.3; ●, calculated from three Cotton effect analysis of observed ORD at pH 0.35; ○, calculated from three Cotton effect analysis of observed ORD at pH 6.3).

Thus, in the region above 190 mμ the optical activity associated with the carboxyl group takes the form of a small, positive Cotton effect. Using a value for $[\phi]_{ext}$ of 2000°, the magnitude of the rotation at the peak (225 mμ), the rotational strength of the transition is calculated to be 3.3×10^{-40} erg cm³ by eq 4 and 5. By way of comparison, the rotational strength of the $n \rightarrow \pi^-$ amide transition for homopolypeptides in the α-helical conformation is approximately 20×10^{-40} erg cm³, as calculated by Carver, *et al.*[14]

B. L-Cysteine. As shown in Figure 1, the presence of a sulfhydryl group on the β-carbon atom results in an increase in the rotations of the negative limb of the carboxyl Cotton effect, producing a marked asymmetry. However, the effect of pH on the ORD curve is similar to that observed with alanine; *e.g.*, at neutral pH the peak of the Cotton effect is blue shifted and slightly reduced in magnitude.

The absorption spectrum of cysteine includes a prominent band at 190 mμ (Figure 3). A similar band is found in the spectrum of β-mercaptoethanol (Figure 3) at 196 mμ, and has also been observed with ethanethiol[23] at 195 mμ. This band is thought to be associated with an $n \rightarrow \sigma^-$ transition of the sulfhydryl group.[24] It is not possible to detect the presence of the weak carboxyl band at 205 mμ because of the overlapping of the sulfhydryl band. Urry, *et al.*,[21] have reported observing an absorption maximum at 283 mμ for cysteine at pH 6.2 which is assigned to a sulfhydryl transition. We have not observed this band in the spectrum of cysteine at acidic pH nor has it been previously reported for cysteine or other sulfhydryl compounds. A possible explanation for these disparate findings might be found in the fact that the band at 283 mμ was observed with solutions from which molecular oxygen had been rigorously excluded.

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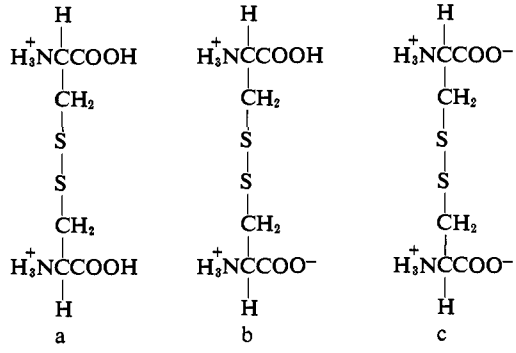
On comparing the ORD and absorption spectra of L-cysteine, it is evident that the 190-mμ sulfhydryl transition is not responsible for the large negative rotations of the cysteine Cotton effect since a Cotton effect centered at 190 mμ would be expected to give rise to an extremum or shoulder at some wavelength greater than 190 mμ. Thus, the marked asymmetry of the Cotton effect appears to reflect the presence of one or more Cotton effects at wavelengths below 190 mμ which are not present with alanine. Whether these are associated with carboxyl or sulfhydryl transitions cannot be ascertained from these data. In any event, the ORD of cysteine in the region investigated is basically similar to that observed for alanine and appears to be dominated by the $n \rightarrow \pi^-$ carboxyl Cotton effect.

C. L-Cystine. The oxidation of the sulfhydryl group of cysteine to form cystine results in a profound change in the rotatory properties, as may be seen by comparing the ORD of L-cystine (Figure 4) with that of L-cysteine (Figure 1). At low pH, the presence of the disulfide bond results in the appearance of a broad trough near 269 mμ and a deep trough at 208 mμ, while a peak is found at 233 mμ. At higher pH's, the peak becomes barely discernible but is not shifted toward shorter wavelengths as was the peak for alanine and cysteine. The magnitude of the short-wavelength trough is even greater at pH 6.3 than at low pH.

It appears that there are two wavelengths at which the rotations of cystine are constant with changing pH: 233 and 204 mμ. Such isorotational points suggest that the ORD curves for pH's in the range investigated arise as linear combinations of two "parent" curves in proportions which are a function of the pH. Since the major effect of changing the pH in the region below pH 7.0 involves the ionization of the carboxyl groups of cystine, it would seem likely that the ORD of cystine

is influenced by the state of ionization of the carboxyl groups and that the "parent" curves are associated with two distinct molecular species: the zwitterionic form in which both carboxyls are completely ionized, and the acidic form in which both are completely un-ionized. This conclusion is not necessarily obvious since there are three molecular species involved.



The ORD of form b can only be a linear combination of the forms a and c in equal amounts if the state of ionization of one carboxyl group has no effect on the optical activity associated with the other. The ORD curve for cystine at pH 1.63 (Figure 4) where the molecule is primarily in the b form is approximately intermediate between the curves for pH 0.35 and 6.3.

Far-ultraviolet ORD curves for cystine in the pH range 0.5–1 which are in qualitative agreement with the present results have been reported by several investigators.[19,25,26] Since the pK_A for the first carboxyl group is near 1.0,[27] it is likely that differences among the curves reflect the different pH's at which the measurements were made.

The absorption spectrum of cystine in the region 180–270 mμ is of value in interpreting the ORD curves. As shown in Figure 2, the spectrum is much more complex than that of alanine or cysteine. It is useful to compare the spectrum of cystine with that of a model disulfide, 2,2′-dithiodiethanol (Figure 2), to help in the identification of the various bands. The spectrum of the model disulfide includes the maximum near 246 mμ which is characteristic of alkyl disulfides,[28] and also a shoulder at 187 mμ[29] indicating the existence of a second disulfide transition. The spectrum of 2,2′-dithiodiethanol in 1 *N* sulfuric acid was identical with that in water. These results are somewhat at variance with those of Legrand and Viennet[30] who reported a distinct maximum near 187 mμ in the absorption spectrum of cystine at low pH. Measurements of the spectrum of cystine in the solid state reported by Preiss and Setlow[31] also do not show an absorption maximum in this region.

The spectrum of L-cystine at low pH is essentially identical with that for dithiodiethanol except in the region between 200 and 235 mμ. At higher pH's the spectrum in the region of the 246-mμ band is practically unchanged. The effect of pH on the absorption of cystine below 240 mμ is qualitatively quite similar to that seen with alanine. The spectrum has two isosbestic points, 240 and 212 mμ, at which the absorption is constant with respect to changes in pH over the pH range investigated. As with the isorotational points observed in the ORD (Figure 3), these isosbestic points suggest that changes in absorption are associated with changes in the state of ionization of the carboxyl groups.

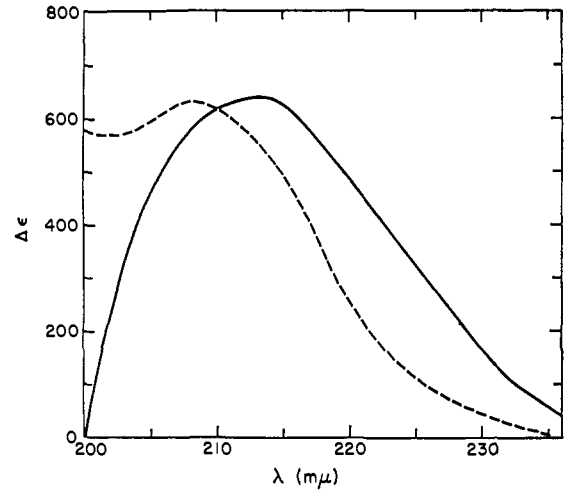


Figure 5. Calculated difference spectrum, ε(cystine) − ε(2,2′-dithiodiethanol) (——, pH 0.35; – – –, pH 6.3).

The "extra" contribution to the spectrum of cystine in the region between 200 and 235 mμ is shown in Figure 5 as a calculated difference spectrum. There is a single band with its peak near 215 mμ at low pH. At neutral pH, the peak is found near 208 mμ. The position and pH dependency of this band indicates that it is associated with the $n \rightarrow \pi^-$ transition of the carboxyl groups, similar to that observed with alanine.

We conclude that cystine has three electronic transitions in the region above 180 mμ which could be optically active: the 246-mμ disulfide transition, a carboxyl transition near 215 mμ (at low pH), and a second disulfide transition near 186 mμ.

Circular dichroism measurements carried out with L-cystine at low pH (Figure 6) reveal two bands and a part of a third band in the region above 200 mμ. The small negative band near 250 mμ has been associated with the disulfide transition by Beychok.[32] The 220-mμ positive band is probably associated with the carboxyl groups, its position being near that for the carboxyl absorption band of Figure 5. In addition, the positive band appears to undergo a blue shift upon ionization of the carboxyl groups such as that observed with the other amino acids. The negative circular dichroism observed below 210 mμ (low pH) indicates that at least one additional band is present.

D. Computer Analysis of the ORD of L-Cystine. In order to determine conclusively whether three Cotton effects associated with the three absorption bands of cystine could be found which would account for the observed ORD curve, a nonlinear, least-squares analysis was carried out. Using the initial parameter esti-

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(31) J. W. Preiss and R. Setlow, *J. Chem. Phys.*, **25**, 138 (1965).
(32) S. Beychok, *Proc. Natl. Acad. Sci. U. S.*, **53**, 999 (1965).

Table I. Computer Analysis of the ORD of L-Cystine[a]

	pH 0.35		pH 6.3		pH 6.3	
	Initial	Final	Initial	Final	Initial	Final
$A_1 \times 10^{-4}$, deg cm^2 dmol^{-1}	−0.0257	−0.0155	−0.0120	−0.0524	−0.0480	−0.0296
λ_1^0, mμ	248.0	252.3	255.0	252.9	252.8	256.4
Δ_1^0, mμ	20.2	19.3	20.0	33.2	33.0	27.6
$A_2 \times 10^{-4}$, deg cm^2 dmol^{-1}	0.183	0.179	...	...	0.120	0.119
λ_2^0, mμ	218.0	217.7	...	...	212.0	204.4
Δ_2^0, mμ	15.0	12.9	...	...	10.0	18.3
$A_3 \times 10^{-4}$, deg cm^2 dmol^{-1}	−0.524	−0.684	−0.722	−0.472	−0.529	−0.722
λ_3^0, mμ	190.3	187.1	183.0	187.1	188.0	185.4
Δ_3^0, mμ	16.0	20.4	10.0	5.5	4.2	11.1
RESMS $\times 10^{-8}$	24.2	0.138	328	10.1	488	0.51
Exptl RESMS $\times 10^{-8}$		0.22		0.60		0.60

[a] For meaning of symbols see text.

mates given in Table I, a fit of the observed curve to within experimental error was obtained for cystine at low pH. The Cotton effect parameters for this fit are given in Table I, and the rotations calculated for this set of Cotton effects are shown in Figure 4. Although various combinations of Cotton effects and background terms were tested in addition to the set shown in Table I, no significant improvement in the fit was obtained. The CD curve corresponding to the calculated ORD curve was calculated from the Cotton effect parameters using eq 1 and is included in Figure 6. In view of the

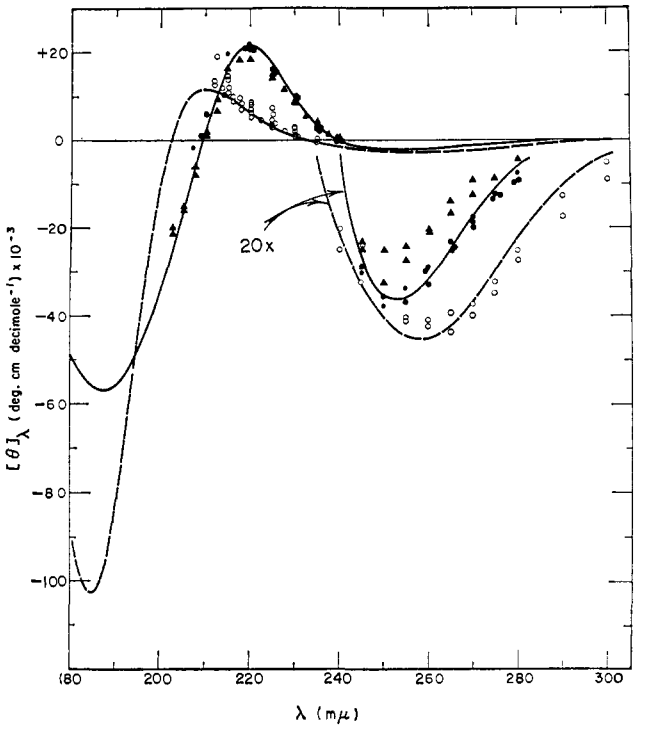

Figure 6. Ellipticity of L-cystine (● and ▲, observed at pH 0.35; ○, observed at pH 6.3; ——, calculated from three Cotton effect analysis of observed ORD at pH 0.35; – – –, calculated from three Cotton effect analysis of observed ORD at pH 6.3).

scatter in the experimentally obtained data, the agreement between the calculated and observed curves is quite acceptable. The calculated curve shows clearly the large negative band near 186 mμ although it had not been possible to observe this band directly.

These results show that the localized nature of CD bands relative to the dispersed nature of Cotton effects may be an advantage on some occasions but a distinct disadvantage on others. Although CD measurements may be superior in allowing separation of various overlapping contributions, the delocalized nature of a Cotton effect provides information about optically active transitions in inaccessible regions of the spectrum which cannot be obtained from CD measurements.

In attempting to fit the ORD of cystine at pH 6.3, sets of two and three Cotton effects were used. As shown in Table I, the RESMS for the two Cotton effect fit was not acceptable in spite of the fact that the observed curve gives the appearance of consisting of only two Cotton effects. With the three Cotton effect fit, an acceptable RESMS was obtained and the calculated rotations, shown in Figure 4, were in satisfactory agreement with the observed values. As with the low pH data, no improvement in the fit was obtained when various types of background terms were added. The CD curve associated with the calculated ORD curve for the solution at neutral pH is given in Figure 6. Legrand and Viennet[30] have reported CD spectra for cystine which generally agree with the calculated curves in Figure 6. They observed that the positive band shifts from 219 to 207 mμ when the pH is raised from *ca.* 1 to *ca.* 13. At low pH, they found a large negative band at 196 mμ. The position of this band does not agree with that for the calculated CD curve of Figure 6, nor with the shoulder observed in the absorption spectrum of cystine and 2,2′-dithiodiethanol near 187 mμ.

With the fitting of the observed ORD curves we have an indication that the resolution of the curves suggested by the location of the absorption and CD bands is correct, and we also have quantitative information which is useful in arriving at an interpretation of the origin and nature of these Cotton effects. A discussion of this point follows the presentation of results of investigations on the optical activity of the disulfide bond in derivatives of cystine.

E. N,N′-Diacetyl-L-cystine. The ORD and CD of N,N′-diacetyl-L-cystine (DAC) indicate that modification of the amino groups of cystine has only minor effects on the rotatory properties of the disulfide group. At pH 0.35, the differences between the ORD of DAC and that of cystine (Figure 7) indicate that the magnitude of the longer wavelength disulfide Cotton effect (which we shall call the *first* disulfide Cotton effect) may be somewhat reduced with DAC relative to that for cystine. The blue shift in the position of the deep trough of DAC relative to that with cystine could be due to a shift in the position of the shorter wavelength

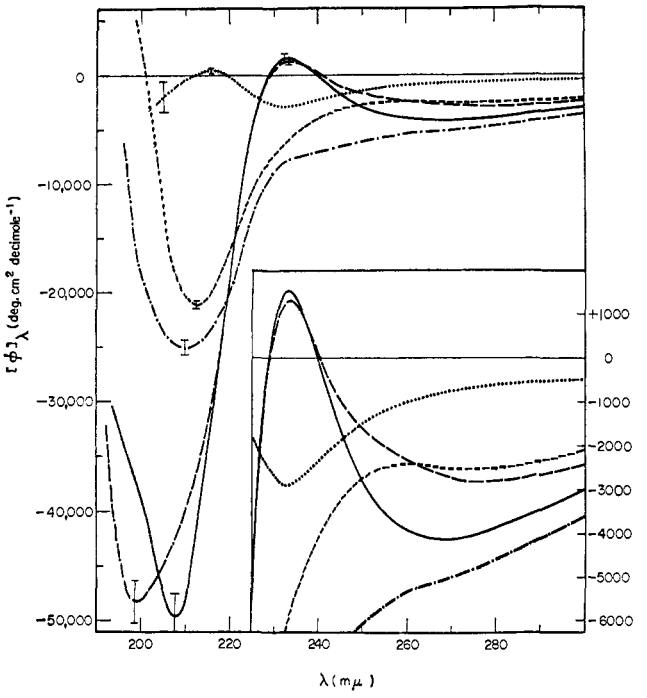

Figure 7. ORD of derivatives of L-cystine (——, L-cystine at pH 0.35; — — —, N,N′-diacetyl-L-cystine at pH 0.35; - - - -, N,N′-diacetyl-L-cystine bismethylamide at pH 6.3; ·-·-·-, oxidized glutathione at pH 3.0; · · · · ·, reduced glutathione at pH 3.0).

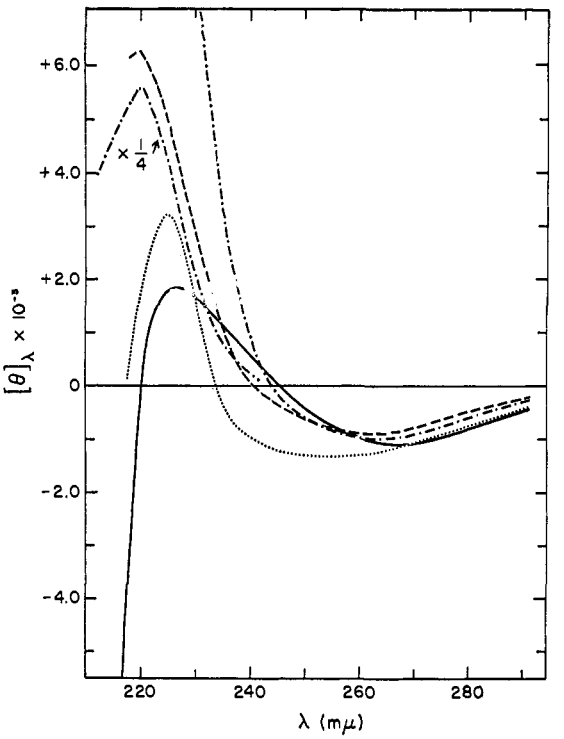

Figure 8. Ellipticity of derivatives of L-cystine (——, N,N′-diacetyl-L-cystine bismethylamide at pH 0.35; - - -, N,N′-diacetyl-L-cystine bismethylamide at pH 6.3; ·-·-·-, N,N′-diacetyl-L-cystine at pH 0.35; · · · · ·, oxidized glutathione at pH 3.0).

Cotton effect (the *second* disulfide Cotton effect), or to a reduction in the half-width of the Cotton effect.

The change in the magnitude of the first disulfide Cotton effect inferred from the ORD of DAC is clearly demonstrated by a similar reduction in the size of the corresponding CD band (Figure 8). In addition to the twofold reduction in the value of $[\theta]_{ext}$ relative to that for cystine, the position of the extremum had been shifted by 10 to 262 mμ. On the other hand, the positive CD band of DAC in the region of 220 mμ is essentially identical with that for cystine at pH 0.35. Although absorption bands associated with both the $n \rightarrow \pi^-$ and $\pi^0 \rightarrow \pi^-$ transitions of the amide groups of DAC lie in the spectral region under investigation,[33] no indication of contributions arising from the amide groups is found in either the ORD or CD curves. Thus, these groups appear to have negligible optical activity in the case of DAC.

F. N,N′-Diacetyl-L-cystine Bismethylamide. The ORD of N,N′-diacetyl-L-cystine bismethylamide (DACMA) is considerably different from the ORD of L-cystine or DAC as shown in Figure 7 for solutions at neutral pH, the changes being found principally in the region where the carboxyl Cotton effect is observed with cystine and DAC. Whether changes in the second disulfide Cotton effects are also responsible, in part, for the reduction in size of the short-wavelength trough cannot readily be determined by simple inspection. At pH 0.35, the ORD of DACMA is similar to that shown in Figure 7, although the 210-mμ trough is slightly larger than it is at neutral pH.

CD measurements with DACMA[34] (Figure 8) at pH 0.35 reveal that the negative band at 262 mμ is practically identical with that for DAC, while a small positive band near 220 mμ is found in place of the much larger band observed in this region with cystine and DAC. In contrast to the carboxyl CD band of cystine, this positive band of DACMA is increased in magnitude when the pH is raised to 6.3. In view of the fact that the formation of DACMA involves the introduction of two additional amide groups into the molecule, it appears likely that the positive CD band of DACMA is associated with the $n \rightarrow \pi^-$ transition of these groups. This is consistent with the report that the $n \rightarrow \pi^-$ absorption band of secondary amides is found in the region 210–220 mμ.[35] The pH effect on this band is probably due to a small degree of protonation of the amide groups at pH 0.35.

The existence of an optically active amide transition suggests the presence of a specific interaction giving rise to a favored orientation of that group in accordance with the general concept of the enhancement of optical activity by the introduction of conformational restrictions.[36] For optical activity resulting from a static electrostatic perturbation, the rotational strength is expected to vary inversely with R^5,[37] where R is the distance between the perturbing atom and the chromophore. A difference in optical activity between the amide groups derived from the carboxyl groups of cystine and those involving the amino groups of the parent molecule could arise either from differences in the degree of conformational restrictions between the two groups or from differences in the distance and orienta-

(33) D. B. Wetlaufer, *Advan. Protein Chem.*, 17, 303 (1962).
(34) Dr. Beychok, who kindly made his Jouan dichrographe available for these measurements, has published a preliminary report[32] of these results.
(35) A. N. Glazer and K. Rosenheck, *J. Biol. Chem.*, 237, 3674 (1962).
(36) W. Kauzmann and H. Eyring, *J. Chem. Phys.*, 9, 41 (1941).
(37) B. J. Litman and J. A. Schellman, *J. Phys. Chem.*, 69, 978, (1965).

Table II. Computer Analysis of the ORD of N,N'-Diacetyl-L-cystine Bismethylamide, pH 6.3

	Initial	Final	Initial	Final
$A_1 \times 10^{-4}$, deg cm^2 dmol^{-1}	−0.01048	−0.01309	−0.01309	−0.01506
λ_1^0, mμ	269.5	263.7	263.7	262.4
Δ_1^0, mμ	24.3	29.0	29.0	30.7
$A_2 \times 10^{-4}$, deg cm^2 dmol^{-1}	0.0100	0.01328	0.01328	0.01619
λ_2^0, mμ	224.0	228.8	228.8	227.6
Δ_2^0, mμ	8.0	20.6	20.6	19.3
$A_3 \times 10^{-4}$, deg cm^2 dmol^{-1}	−0.1795	−0.1964	−0.1964	−0.309
λ_3^0, mμ	204.6	203.8	203.8	199.2
Δ_3^0, mμ	9.1	10.2	10.2	13.1
$A_4 \times 10^{-4}$, deg cm^2 dmol^{-1}	...	...	−0.008	0.1308
λ_4^0, mμ	...	...	185.0	181.3
Δ_4^0, mμ	...	...	10.0	7.3
RESMS $\times 10^{-8}$	18.2	1.52	4.51	0.362
Exptl RESMS $\times 10^{-8}$		0.35		0.35

tion of perturbing atoms relative to the two types of amides.

G. Computer Analysis of the ORD of DACMA. To determine the parameters of the disulfide Cotton effects in DACMA, a computer analysis was carried out using three Cotton effects having the parameter values given in Table II. Although a convergent solution was obtained, the deviations between calculated and observed rotations in the region of the deep trough near 210 mμ were greater than experimental error. Therefore, a fourth Cotton effect fixed at 185 mμ was added to approximate a background contribution. The fit obtained with the parameter values given in Table II for this set of Cotton effects had an acceptable RESMS, and a point by point inspection of the deviations of the calculated values from the observed values revealed that the fit was generally quite good (Figure 9). A comparison of the Cotton effect

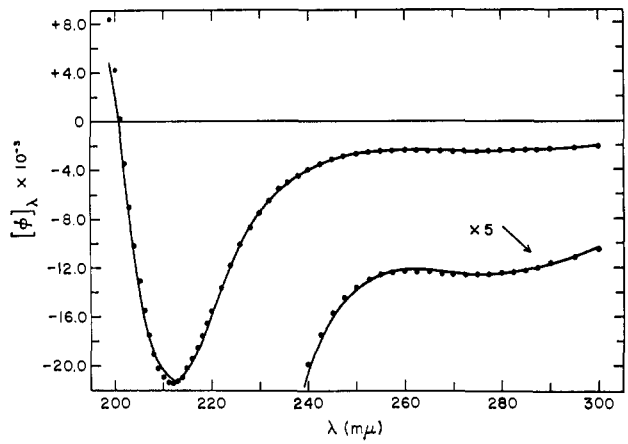

Figure 9. ORD of N,N'-diacetyl-L-cystine bismethylamide (●, calculated rotations from four Cotton effect analysis of observed ORD at pH 6.3; ——, observed ORD at pH 6.3).

parameters for the four Cotton effect fit of the ORD of DACMA at pH 6.3 (Table II) with those for cystine at the same pH (Table I) indicated that the first disulfide Cotton effect (A_1, λ_1^0, Δ_1^0) is red shifted by *ca.* 6 mμ, and its rotational strength is diminished by nearly a factor of 2 when the amino and carboxyl groups of cystine are incorporated into amide groups. The second disulfide Cotton effect (A_3, λ_3^0, Δ_3^0) is red shifted 14 mμ, and its rotational strength reduced by nearly 60%. However, this comparison does not permit distinguishing between the effect of modifying the amino groups and that of modifying the carboxyl groups.

H. Rotatory Properties of Reduced DACMA. The ORD curve of N-acetyl-L-cysteine methylamide (reduced DACMA) has the form of a single negative Cotton effect with its trough at 225 mμ in contrast to the complex ORD curve of DACMA (Figure 10). The CD curve of reduced DACMA (Figure 11) has a single negative band whose extremum appears to lie near 210 mμ. Both the negative disulfide band near 260 mμ and the positive band at 220 mμ which were found with DACMA are missing. Since the sulfhydryl group does not have any transitions in this region, this band may be assigned to an n → π* amide transition, probably that of the carboxyl-derived amide group since the oxygen atom of this group is closer to the asymmetric center of the molecule.

From this comparison of the ORD and CD curves of reduced DACMA with those of DACMA, it is evident that the presence of the disulfide bond has a marked effect on the n → π* amide transition and its optical activity, resulting in both a substantial red shift in position and a change in sign of the Cotton effect or CD band. Bergson, *et al.*,[28] have reported similar red shifts of the n → π* carboxyl transition of disulfide-containing carboxylic acids. Similarly, the position of the carboxyl Cotton effect of cystine (λ_2^0 in Table I; also see section C) is shifted more than 10 mμ toward longer wavelengths relative to the position of this Cotton effect for alanine. The change in sign of the amide Cotton effect may be interpreted (in terms of a general octant rule) as a difference in orientation of the perturbing group responsible for the optical activity of the amide group of DACMA compared with reduced DACMA. Such a difference could result in a change in the sign of the Cotton effect. On the other hand, a difference in the type of perturbation (*e.g.*, incomplete screening of nuclei, polarizability, or static charge) could lead to a change in the sign without involving a change of octant.

I. Glutathione. The ORD and CD curves of the naturally occurring disulfide, oxidized glutathione (GSSG), are given in Figures 7 and 8, respectively. Although its presence cannot be verified from the ORD curve, the CD curve[34] shows that the optical activity of the first disulfide transition of GSSG is much the same as with DACMA. The broadening of the band is peculiar to GSSG and could indicate the presence of a

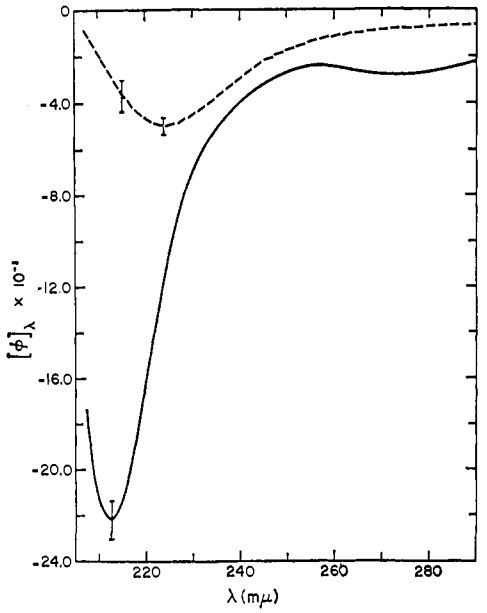

Figure 10. ORD of N,N′-diacetyl-L-cystine bismethylamide (——, pH 0.1) and N-acetyl-L-cysteine methylamide (- - -, pH 0.1).

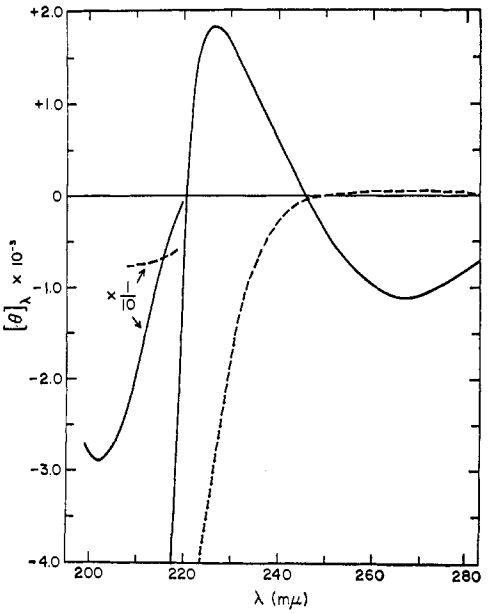

Figure 11. Ellipticity of N,N′-diacetyl-L-cystine bismethylamide (——, pH 0.1) and N-acetyl-L-cysteine methylamide (- - -, pH 0.1).

second unidentified band in the region near 240 mμ. The positive band found near 225 mμ is similar in position and magnitude to that observed with DACMA. The deep trough at 210 mμ in the ORD is equivalent in size to the trough of DACMA at pH 0.35. These similarities between the rotatory properties of DACMA and GSSG are not surprising since the peptide bond between the Glu and Cys residues is undoubtedly too far from the asymmetric center of the glutamate residue to be influenced by it. Hence, the peptide bonds of the molecule probably are influenced primarily by the Cys residue alone. The ORD of GSSG might be expected to include a Cotton effect associated with the α-carboxyl groups of the glutamate residues, but any such contribution is evidently too small to produce significant deviations relative to the ORD of DACMA.

The ORD of reduced glutathione (Figure 7) is similar to that of reduced DACMA, having the form of a simple negative Cotton effect. Thus, the same change of sign and blue shift of position of the $n \rightarrow \pi^-$ amide transition occurs upon reduction of GSSG as with DACMA.

J. The Disulfide Contribution to the Optical Activity of Proteins. From the results presented above, we wish to estimate the importance of the disulfide contribution to the ORD or CD of disulfide-containing proteins. In Table III, the value of $[\phi]_{589}^{SS}$ is given as −436°, as calculated on the basis of the disulfide Cotton effect parameters obtained from the computer fit of the ORD of DACMA (Table II). For a protein with a disulfide content of 3% (three S–S bonds per hundred residues) the contribution to $[R']_{589}$ would be of the order of −13°. Although this contribution may not account for the entire change in $[R']_{589}$ upon reduction of the disulfide bonds of a protein, it should be taken into consideration when analyzing the results of such experiments.

The magnitude of the disulfide contribution as determined from the ORD of DACMA is of the same order of magnitude as that determined by Würz and Haurowitz.[38] However, their conclusion that the observed changes in $[\phi]_{589}^{prot}$ upon reduction of S–S bonds are due only to the disulfide contribution must be restricted to those changes observed on going from a disulfide-containing protein in a denaturing solvent to a reduced (or oxidized) protein in the same solvent. Investigations of a number of proteins, including human serum albumin[39] and ribonuclease,[40] have shown a considerable increase in the magnitude of $[\alpha]_{589}$ or $[\phi]_{589}$ for the intact protein in these denaturing solvents over the value in neutral buffers. The *subsequent* change which accompanied cleavage of the disulfide bonds was often small relative to these initial changes. Moreover, from studies of synthetic disulfide-containing polypeptides, we have reported[4] evidence which suggests that the disulfide contribution to the optical activity of proteins may be greatly reduced or eliminated when the

Table III. Characteristics of the Disulfide Optical Activity of N,N′-Diacetyl-L-cystine Bismethylamide

Disulfide Cotton Effect Parameters[a]	
$R_1 \times 10^{40}$, erg cm³	−1.641 ± 0.235
$A_1 \times 10^{-4}$, deg cm² dmol⁻¹	−0.01506 ± 0.00216
λ_1^0, mμ	262.3 ± 2.9
Δ_1^0, mμ	30.7 ± 1.8
$R_2 \times 10^{40}$, erg cm³	−33.7 ± 8.2
$A_2 \times 10^{-4}$, deg cm² dmol⁻¹	−0.310 ± 0.075
λ_2^0, mμ	199.2 ± 1.8
Δ_2^0, mμ	13.1 ± 1.2
Disulfide Rotations[b] and Ellipticities[c]	
$[\phi]_{589}^{SS}$	−436
$[\phi]_{211}^{SS}$	-24.2×10^3 (negative extremum)
$[\phi]_{187}^{SS}$	$+26.6 \times 10^3$ (positive extremum)
$[\theta]_{262}^{SS}$	-1.138×10^3
$[\theta]_{199}^{SS}$	-41.8×10^3

[a] From computer analysis of the ORD of DACMA. [b] In degrees. [c] Calculated on the basis of the disulfide Cotton effect parameters.

(38) H. Würz and F. Haurowitz, *J. Am. Chem. Soc.*, 83, 280 (1961).

(39) G. Markus and F. Karush, *ibid.*, 79, 134 (1957).

(40) W. Harrington and M. Sela, *Biochim. Biophys. Acta*, 31, 427 (1959).

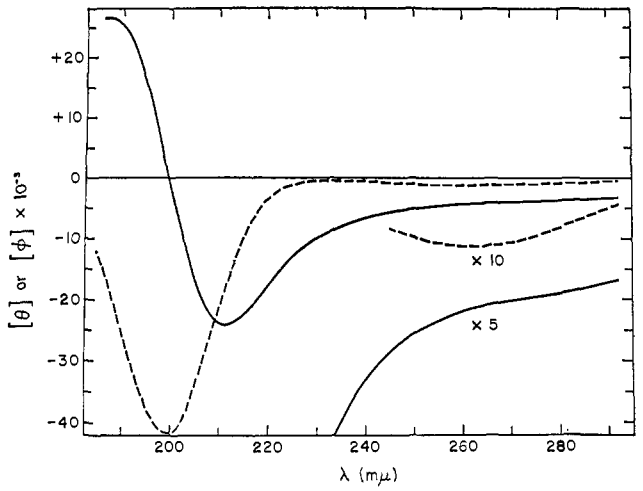

Figure 12. Rotatory properties of the disulfide chromophore of N,N'-diacetyl-L-cystine bismethylamide calculated from Cotton effect parameters derived by computer analysis (——, ORD; – – –, ellipticity).

protein is unfolded and in a random conformation. Thus, the significance of cleaving the disulfide bonds lies not so much in the changes in optical rotation which accompany the cleavage but in the fact that the larger conformational changes resulting from the change of solvent may no longer be reversible, as is often the case when the S–S bonds are intact.

In the far-ultraviolet spectral regions it is of interest to know whether the disulfide Cotton effects can be observed in the ORD of proteins. In Figure 12, the ORD and CD curves for the disulfide group are given as calculated from the Cotton effect parameters obtained from the computer fit of the ORD of DACMA. It is clear that the most significant contribution to the ORD is made by the second disulfide Cotton effect, the 260-mμ Cotton effect being so weak that it can hardly be observed. On the other hand, both contributions can be observed in the CD curve. The significance of these contributions to the ORD or CD curves of disulfide-containing proteins will, of course, depend on the relative magnitudes of contributions from other groups which may overlap with the disulfide contributions. We have not attempted to estimate the significance of the indirect effect of the disulfide, *i.e.*, the modification of the optical activity of other transitions such as those of the amide groups because this may well depend on additional factors such as the conformation of the polypeptide chain. It is apparent, however, that this contribution is of minor importance relative to the direct S–S contribution in the case of DACMA.

In spite of the large rotational strength of the 200-mμ disulfide Cotton effect, its contribution to the value of $[R']_{210}$ (where the contribution is largest) will be of the order of only -500 to $-1000°$ for proteins containing two to four S–S bonds per hundred residues. The presence of strong, overlapping peptide Cotton effects would make it difficult to observe the trough of a disulfide Cotton effect in this region. Thus, no evidence of this Cotton effect is observed in the far-ultraviolet ORD curves of several disulfide-containing proteins. However, in the case of the small cyclic peptides, 8-L-arginine-vasotocin and 8-L-ornithine-vasopressin, the 200-mμ disulfide Cotton effect has been observed[4] to account for the major part of the ORD curves in the far-ultraviolet spectral regions. In investigations of the CD of disulfide-containing proteins, the 260-mμ disulfide band has been observed with human serum albumin,[41] insulin,[32,42] and 8-L-arginine-vasotocin,[4] as it was with DACMA and GSSG. On the other hand, it was not possible to differentiate the 200-mμ disulfide band from the peptide bands when the far-ultraviolet CD of serum albumin was investigated.[41]

These observations lead us to conclude that disulfide Cotton effects will not generally cause significant errors in the estimation of the helical content of disulfide-containing proteins from ultraviolet ORD data. However, it is conceivable that under conditions where the other Cotton effects of the molecule, such as the peptide Cotton effects associated with the random coil and α-helix conformations, cancel each other in large part, the disulfide trough at 210 mμ could be observed or at least account for a substantial portion of the observed rotation in the region. For example, using the Cotton effect parameters obtained for the α-helix and random coil by Carver, Shechter, and Blout,[14] we have calculated that the contributions of the disulfide Cotton effects in the region near 210 mμ will exceed 10% of the values of [R'] for helix contents between 25 and 40%, for a polypeptide containing two S–S bonds per hundred residues.

K. Qualifications of the Use of DACMA as a Model. In addition to the question of whether DACMA is a suitable model for cystine in a peptide chain, this extrapolation of the results of our investigation of the optical activity of DACMA to the ORD or CD of proteins must be subject to certain additional qualifications. First, it was noted that the presence of the disulfide bond apparently has a perturbing effect on other Cotton effects of the molecule. This was observed with the $n \rightarrow \pi^-$ amide Cotton effect with DACMA and GSSG as well as with the $n \rightarrow \pi^-$ transition of the carboxyl groups of cystine and DAC, where the carboxyl Cotton effects were found at longer wavelengths and were somewhat larger than those for alanine or cysteine.

A second point pertains to the relationship of the dihedral angle of the S–S group to the magnitude and sign of the disulfide Cotton effects. Schellman[22] has noted that, because of the geometry of the disulfide bond, the group is inherently asymmetric and that two distinct diastereoisomers can exist. He suggested that the S–S group is intrinsically optically active as a result of this asymmetry, with the ORD or CD of the two isomers being equal in magnitude but opposite in sign. When the R groups of a disulfide, RSSR, are each symmetrical, the two isomeric forms of the S–S group would have equal energies resulting in equal numbers of the two isomers, and no net optical activity. But with asymmetric side chains, such as with cystine, Schellman suggested that one isomer might be energetically favored over the other, giving rise to an imbalance in the relative numbers of the two isomers and. hence, to a net optical activity. Beychok[32] has extended this hypothesis to S–S bonds in proteins where, because of the small

(41) M. Legrand and R. Viennet, *Compt. Rend.* **259**, 4227 (1964).
(42) M. Grosjean and M. Tari, *ibid.*, **258**, 2034 (1964).

number of S–S bonds per molecule, the distribution of isomers might be significantly different from that of cystine or other small disulfides, especially as any given S–S bond in a native protein always exists in the same isomeric form. The result would be a marked variation in the magnitude and possibly the sign of the disulfide Cotton effects or CD bands among various disulfide-containing proteins. Since the model compounds included in this investigation did not have fixed dihedral angles, it is not possible to unequivocally categorize the changes in disulfide optical activity associated with the various modifications as arising from changes in the dihedral angle or distribution of isomers or from different environmental perturbations.

In consideration of these proposals, it may be noted that Moscowitz[43] has calculated that the rotational strength of an inherently asymmetric chromophore should be of the order of 10^{-38} erg cm^3, while the rotational strength of a chromophore in which optical activity arises through asymmetric environmental perturbations would be expected to be considerably smaller in magnitude. The rotational strength (Table III) of the first disulfide transition of DACMA (at 262 mμ) is only -1.6×10^{-40} erg cm^3, whereas the value for the second transition (at 199 mμ) is -33.7×10^{-40} erg cm^3, a value which approaches that calculated by Moscowitz. The rotational strength of the short-wavelength disulfide transition is even larger in the case of cystine, being -75×10^{-40} and -89×10^{-40} at pH 0.35 and 6.3, respectively (calculated from the values of A_s^0 given in Table I, using eq 5). In the absence of measurements of this Cotton effect, the low rotational strength of the long-wavelength disulfide transition might have been explained in terms of a nearly equal distribution of the two possible disulfide isomers such that the contributions from the two isomers canceled each other in large part. However, if the rotational strength of the short-wavelength transition truly reflects the asymmetry of the disulfide bond it signifies a distribution which markedly favors one isomer. These results suggest that, whereas the optical activity of the short-wavelength disulfide transition may be sensitive primarily to the asymmetry of the S–S group and, hence, to the dihedral angle, the optical activity of the long-wavelength transition may arise largely as a result of interactions with vicinal atoms and solvents.

A consideration of the assignments of the two absorption bands associated with these disulfide Cotton effects provides some insight into the reasons for this marked difference in optical activity. Starting with sp^3-type orbitals for the bonding electrons and the two unshared electron pairs on each sulfur atom, Bergson[44] used an LCAO approach to form a system of two π^+ and two π^- orbitals occupied by the four unshared pairs of the disulfide group. At dihedral angles near 90° the two π^- orbitals have nearly the same energy. The same is true of the two π^+ orbitals, with the energy of the bonding orbitals being considerably lower than that for the antibonding orbitals. Transitions of these electrons are considered to involve promotion to a vacant σ^- orbital. Although the σ^- orbital is insensitive to the dihedral angle of the disulfide group the energies of the π^- and π^+ orbitals vary considerably with the dihedral angle due to variations in the degree of overlap in the sp^3 orbitals which make up the molecular orbitals. This variation was found to qualitatively account for the red shift in the position of the disulfide absorption band from its normal position near 250 mμ when the dihedral angle is decreased from approximately 90° to much smaller values. This band was assigned to the $\pi^- \rightarrow \sigma^-$ transition. On the basis of Bergson's analysis, the disulfide band observed near 190 mμ with cystine and 2,2′-dithiodiethanol would be a $\pi^+ \rightarrow \sigma^-$ transition.

It is clear that the electrons in the π^- orbitals are highly localized and have the characteristics of unshared electrons, especially at dihedral angles near 90° where the overlap necessary for the molecular orbital formation is minimal. The optical activity associated with transitions involving electrons thus confined to one or the other of the sulfurs will not directly reflect the asymmetry of the S–S group but will be characteristic of a symmetrical chromophore having a large magnetic transition dipole moment and a weak electric transition dipole moment arising from asymmetric perturbations of an electrically forbidden transition. These perturbations may arise from other groups of the molecule as well as from the other half of the disulfide. On the other hand, the electrons in the π^+ orbitals, being weakly bonding electrons, will be delocalized over the asymmetric S–S group. Transitions involving these electrons will display optical activity which is characteristic of an inherently asymmetric chromophore and is directly related to the geometry of the disulfide bond.

It should be noted that the induced optical activity of the 250-mμ transition would not be expected to be of equal magnitude and opposite sign for the two disulfide isomers, nor would the optical activity of this transition necessarily vanish when the S–S group assumed a planar configuration. Since it has been demonstrated[28] that the energy of the 250-mμ transition is a function of the dihedral angle, the position of the associated absorption band (or Cotton effect or CD band) appears to be a better indicator of changes in the disulfide bond configuration than is the rotational strength of this transition. However, it is quite possible that the energy of the transition may be influenced by factors in addition to the dihedral angle. Thus, we conclude that the interpretation of the magnitude and sign and, to some extent, the position of the 260-mμ disulfide Cotton effect or CD band in terms of the configuration of the group must be undertaken with some reservations.

Although it is likely that the rotational strength of the short-wavelength S–S transition is a more reliable indicator of the disulfide configuration, its usefulness in investigations of protein structure is limited by the difficulties in observing the Cotton effect or CD band in the presence of large contributions from the overlapping peptide transitions. With the application of more refined analytical techniques, such as the computer analysis developed by Carver, the characterization of this Cotton effect in the ORD of proteins may become possible.

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(43) A. Moscowitz, *Tetrahedron*, 13, 48 (1961).
(44) G. Bergson, *Arkiv Kemi*, 18, 409 (1961).

ments. Finally, we wish to thank Mr. J. R. Parrish, Jr., for his interest and for helpful criticisms of this work. We acknowledge with thanks the financial support provided by the National Institutes of Health in the form of a predoctoral fellowship to D. L. C. and by U. S. Public Health Service Grant No. AM07300 to E. R. B.

Communications to the Editor

The Course of Allylic Coupling Reactions Involving Allylnickel Complexes

Sir:

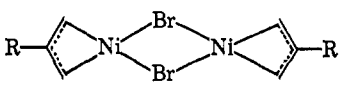
The reaction of π-allylnickel(I) halide complexes with a wide variety of organic halides to form *cross-coupling* products selectively has recently been reported.[1] We describe herein the sharply contrasting and relatively complex behavior of *allylic halides* toward π-allylnickel complexes and its relevance to the intermolecular[2] and intramolecular[3-5] coupling of allylic halides by nickel carbonyl.

Although solutions of pure[1] π-allylnickel(I) bromide (**1**) (blood red in color) in tetrahydrofuran or glyme solvents are *stable*[6] at least for several days at 25°, the addition of allyl bromide (2 moles/mole of **1**) at 25° results in quantitative formation of biallyl within a few minutes.[7] That this latter reaction is not a simple cross-coupling process is indicated by the fact that treatment of the allyl complex **1** with *methallyl* bromide (2 moles/mole of **1**) in tetraglyme leads in high yield (>95%) to a *mixture* of all three possible coupling products, biallyl (35%), allylmethallyl (25%), and bimethallyl (40%); similarly, reaction of the methallyl complex **2** with allyl bromide produces (>95%) a *mixture* of biallyl (22%), allylmethallyl (53%), and bimethallyl (25%).[8] Nonspecific coupling also results when allylic iodides, chlorides, or tosylates are substituted for bromides in these reactions or with dimethylformamide as solvent. A major reason for this nonspecificity is the occurrence of a rapid exchange reaction according to eq 1 (A = allyl) under normal coupling conditions. Experimentally the exchange can be demonstrated by slow addition (by motor-driven syringe) of the complex **1** (1 mmole) in dimethylformamide to methallyl bromide (30 mmoles) in the same solvent at 22°; this affords a mixture of the expected hydrocarbon coupling products (0.32 mmole of C_6, 0.8 mmole of C_7, and 0.8 mmole of C_8) and, *in addition*, *allyl bromide* (0.68 mmole), with excess methallyl bromide remaining unchanged. A strictly analogous experiment in which complex **2** was added to allyl bromide afforded a mixture of the expected hydrocarbon coupling products (1.36 mmoles of C_6, 0.6 mmole of C_7, and 0.04 mmole of C_8) and *methallyl bromide* (1.2 mmoles), as well as unchanged allyl bromide. The rapid exchange described by eq 1 can even be demonstrated in toluene as solvent under conditions where the coupling reaction is very slow.[7] Ten minutes after mixing 1 mole of **2** with 2 moles of allyl bromide in toluene at 25°, methallyl bromide was formed in substantial amount (allyl:methallyl bromide = 2.3 by vpc analysis).

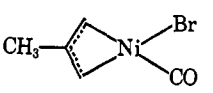
$$[\pi\text{-A}^1\text{NiBr}]_2 + \text{A}^2\text{Br} \rightleftharpoons [\pi\text{-A}^2\text{NiBr}]_2 + \text{A}^1\text{Br} \quad (1)$$

It has also been possible to show that the reaction between allylic halides and nickel carbonyl to form π-allylnickel(I) complexes is *easily reversible* and that this is a factor of considerable importance in the allylic coupling reaction with nickel carbonyl. Introduction of carbon monoxide to a mixture of π-methallylnickel(I) bromide and methallyl bromide in tetrahydrofuran results in a substantial acceleration of allylic coupling to bimethallyl. Indeed, carbon monoxide causes the conversion of π-methallylnickel(I) bromide in tetrahydrofuran solution to bimethallyl even in the *absence* of methallyl bromide, according to eq 2. Toluene solutions of π-methallylnickel(I) bromide rapidly absorb one molecule of carbon monoxide per nickel atom to form complex **3**,[9] which reacts with additional carbon monoxide more slowly to give (among other products) methallyl bromide and nickel carbonyl. In addition, when reaction 2 is carried out under coupling conditions at 0° in tetraglyme in the presence of carbon monoxide

$$[\pi\text{-MeANiBr}]_2 + 4\text{CO} \longrightarrow (\text{MeA})_2 + \text{Ni(CO)}_4 + \text{NiBr}_2 \quad (2)$$



1, R = H
2, R = CH_3



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(1) E. J. Corey and M. F. Semmelhack, *J. Am. Chem. Soc.*, **89**, 2755 (1967). See also E. J. Corey and G. H. Posner, *ibid.*, **89**, 3911 (1967), for another cross-coupling method.

(2) See (a) Belgian Patent 448,844 (I. G. Farbenind) (1943); *Chem. Abstr.*, **41**, 6576 (1947); (b) I. D. Webb and G. T. Borcherdt, *J. Am. Chem. Soc.*, **73**, 2654 (1951).

(3) E. J. Corey and E. Hamanaka, *ibid.*, **86**, 1641 (1964); **89**, 2758 (1967).

(4) E. J. Corey and E. K. W. Wat, *ibid.*, **89**, 2757 (1967).

(5) E. J. Corey and M. F. Semmelhack, *Tetrahedron Letters*, 6237 (1966).

(6) All transformations with organonickel compounds were conducted under an argon atmosphere with rigorous exclusion of oxygen.

(7) The rate of formation of π-allylnickel(I) complexes such as **1** from allylic halides and nickel carbonyl is not nearly as sensitive to solvent change as is the coupling reaction. For example, at 50° in benzene formation of complex **1**, though slower than in tetrahydrofuran or glyme solvents, is complete in a few hours; however, the extent of coupling reaction of **1** with allyl bromide is only slight under these conditions. For this reason the preparation of complexes such as **1** is best carried out with hydrocarbon solvents.

(8) Product analyses were performed by vapor phase chromatography (vpc) using columns containing LAC 446 or 728 on Chromosorb P at 75–100° with an F&M Model 609 or 810 unit with flame-ionization detector. Toluene was used as a standard; reproducibility of these results was ±3%.

(9) Complex **3** has been obtained only in solution, since attempted isolation results in loss of carbon monoxide with formation of π-methallylnickel(I) bromide (**2**). Solutions of **3** in benzene exhibit infrared absorption due to carbonyl at 4.80 and 4.88 μ and nmr singlet peaks at 1.48 (downfield from internal tetramethylsilane) (3 H), 2.35 (2 H), and 3.65 ppm (2 H); they do not show the nmr peaks characteristic of **2** at 1.37, 1.60, and 2.37 ppm (ratio, 2:3:2).