Circular Dichroism of Liver Alcohol Dehydrogenase Complexes with Auramine O†

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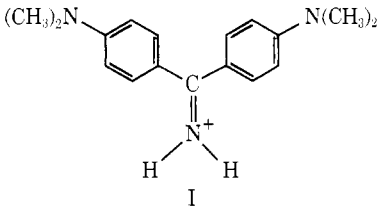
ABSTRACT: The complex of auramine O with horse liver alcohol dehydrogenase exhibits circular dichroism (CD) associated with the dye absorption bands. The sterically required nonplanarity of auramine O makes it a dissymmetric chromophore, and presumably only one enantiomer is bound to the enzyme. The CD of the bound dye thus reflects the intrinsic rotational strengths of the dye transitions and coupling with protein chromophores. Comparison of the observed CD spectra with theoretical calculations indicates that the enantiomer bound has its phenyl rings twisted in a left-handed sense through an angle probably not exceeding 45°. Ternary complexes of the auramine O–liver alcohol dehydrogenase with NAD^+, NADH, and coenzyme fragments (adenosine-5′-P, adenosine-5′-PP, adenosine-5′-PP-ribose) were also studied. Only minor changes in the dye CD bands were observed on binding coenzyme fragments. Large changes were seen when the complete coenzymes were bound. Although a direct interaction between the coenzyme and the dye cannot be completely excluded, arguments are presented which favor a conformational change in the liver alcohol dehydrogenase triggered by the binding of the intact coenzyme.

Auramine (I) is a cationic diphenylmethane dye with the structure



I

Auramine has been shown recently to bind in a highly specific manner to horse liver alcohol dehydrogenase[1] (referred to as the dehydrogenase) at two independent sites with association constants of about 1×10^5 M^{-1} (Conrad *et al.*, 1970). Ternary complexes of liver alcohol dehydrogenase and auramine O with the coenzymes NAD^+ and NADH and with coenzyme fragments (AMP, ADP, and ADPR) have also been described (Conrad *et al.*, 1970; Heitz and Brand, 1971). These ternary complexes are each characterized by a higher association constant for auramine O than that for the binary complex. The binding of coenzymes also results in an alteration of the auramine O fluorescence emission spectrum that is not effected by the fragments. The properties of these complexes (Heitz and Brand, 1971) and kinetic studies (Sigman and Glazer, 1972) lead to the conclusion that the dye-binding site overlaps the substrate site on the enzyme surface.

On the basis of their investigations, Heitz and Brand (1971) have proposed a topographical map of the active site in the dehydrogenase. According to their scheme, coenzymes are bound in an open conformation with distinct sites for the oxidized and reduced forms of the nicotinamide ring. Adjacent to the latter is the substrate-binding site and the dye-binding site.

Although it is generally represented as a planar structure, auramine is in fact sterically prevented from assuming this conformation due to interference between the ring protons ortho to the imino carbon. This steric interference is most efficiently relieved by a conrotatory twist of the phenyl rings. This is illustrated in the photograph of a molecular model shown in Figure 1.

X-Ray and nuclear magnetic resonance (nmr) data show that this type of conformation exists in closely analogous molecules, benzophenone (Lobanova, 1968) and 1,1-diphenylethylene (Rabinovitz *et al.*, 1969).

The conrotatory twist required of the phenyl rings means that auramine O is an inherently dissymmetric chromophore. Presumably, the topography of the dye-binding site is such that only one specific conformer from this racemic distribution can bind to the enzyme. This conformer will be defined by a particular direction and magnitude of ring twist. Through its sensitivity to conformation, circular dichroism is uniquely well suited to provide information about which of the possible auramine conformers is actually bound to the dehydrogenase. This problem will be addressed here by comparing experimental spectra with those obtained from theoretical calculations. The question of possible conformational changes in the dehydrogenase attending the binding of coenzyme, which was not completely resolved in the earlier fluorescence studies due to the proximity of the dye-binding site to the active site (Conrad *et al.*, 1970; Heitz and Brand, 1972), will also be considered in relation to the CD spectra generated by the various auramine O–liver alcohol dehydrogenase complexes.

Materials and Methods

Reagents. Crystalline liver alcohol dehydrogenase was purchased from Sigma Chemical Co., St. Louis, Mo., and dialyzed against 0.1 M sodium phosphate buffer at pH 7.4 as described by Brand *et al.* (1967). Enzyme concentration was determined from the absorbance at 280 nm, using a molar extinction co-

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[1] Abbreviations used are: ADPRib, adenosine diphosphoribose; CD, circular dichroism; PPPSCF, Pariser–Parr–Pople self-consistent field; CT, charge transfer.

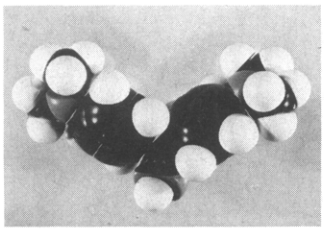

FIGURE 1: Photograph of a molecular model of auramine O.

efficient of 3.54×10^4 M^{-1} cm^{-1} (Conrad *et al.*, 1970). All the nucleotides (NAD, NADH, AMP, ADP, and ADPRib) were also obtained from Sigma and used without further purification.

Auramine O was obtained from Allied Chemicals, New York, N. Y., and purified by successive recrystallizations from acetonitrile until the absorption spectra of consecutive products were identical. The spectral properties of the purified dye were identical with those reported by Conrad *et al.* (1970).

Spectroscopic Methods. Absorption spectra were obtained with a Cary 14 recording spectrophotometer. Circular dichroism spectra were recorded on a Jasco ORD/UV 5 spectropolarimeter with a Sproul SS-20 CD modification.

The absorption and CD spectra were simultaneously resolved into Gaussian components using a six-channel Du Pont 310 curve resolver. The problem of obtaining a unique set of Gaussian bands from an experimental CD spectrum was approached by resolving it together with the corresponding absorption spectrum, and then requiring that the CD bands representing electronically allowed transitions have the same shape and spectral location as the corresponding absorption bands (Moffitt and Moscowitz, 1959). The CD bands representing weak, electrically forbidden transitions were then adjusted to achieve an optimal fit with the experimental trace. This procedure still left some uncertainty in the location, magnitudes, and shapes of the electrically forbidden circular dichroic bands, but this was not considered a serious difficulty.

To render a given pair of absorption and CD spectra into a suitable form for this simultaneous resolution, both experimental tracings were transcribed onto a single graph with the aid of a Calcomp computer plotting routine.

The oscillator strengths, f, and rotational strengths, R, of the resolved absorption and CD bands were calculated in the usual manner (see, for example, Moscowitz, 1960) according to the equations

$$f = 4.62 \times 10^{-9} \epsilon_a^0 \Delta' / \lambda_a^2$$

$$R = 1.63 \times 10^{-4} [\theta_a^0] \Delta' / \lambda_a$$

where λ_a is the wavelength of the band center, ϵ_a^0 and $[\theta_a^0]$ are the values of the extinction coefficient and molecular ellipticity, respectively, at this wavelength, and Δ' is the bandwidth when $\epsilon_a(\lambda) = \epsilon_a^0/2$ or $[\theta_a(\lambda)] = [\theta_a^0]/2$. The rotational strength is given in units of Debye–Bohr magnetons (DBM = 0.9273×10^{-38} cgs unit).

The molecular ellipticity was calculated using the formula (Moscowitz, 1960)

$$[\theta] = 100\psi/lm$$

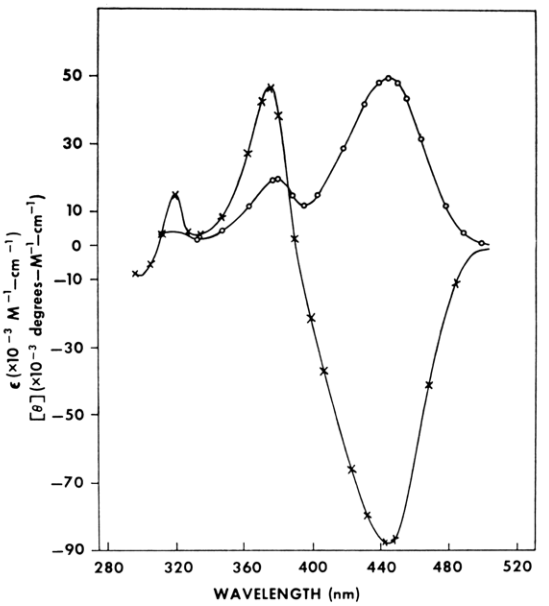

FIGURE 2: Absorption spectrum (○) and CD spectrum (×) of auramine O bound to liver alcohol dehydrogenase. Absorption spectrum: 1.18×10^{-5} M auramine O and 6.4×10^{-4} M liver alcohol dehydrogenase. CD spectrum: 4.76×10^{-5} M auramine O and 2.30×10^{-5} M liver alcohol dehydrogenase. The concentration of bound auramine O was determined as indicated in Methods. ϵ = molar extinction coefficient and $[\theta]$ = molar ellipticity. All spectra were run in 0.1 M sodium phosphate buffer, pH 7.4.

where ψ is the measured ellipticity in degrees, m is the molar concentration of bound auramine, and l is the path length in centimeters.

The molar concentrations of liver alcohol dehydrogenase-bound auramine were calculated for the binary complex and for the various ternary complexes by using the association constants for these complexes obtained by Heitz and Brand (1971).

The CD spectra of the ternary complexes were each obtained by first tracing a spectrum of the binary complex as a standard for comparison, then adding saturating amounts of the particular nucleotide and tracing a second spectrum over the original.

The data from all the runs with the binary complex were averaged to obtain the reported rotational strength values. The CD data for each ternary complex were evaluated relative to the binary spectrum provided by that run. Finally, these relative values were multiplied by the data averages compiled for the binary complex to obtain the reported rotational strength values for the ternary complex.

Theoretical Methods. The molecular orbital calculations were carried out using a Pariser–Parr–Pople self-consistent field approach, as described by Weiss *et al.* (1965). The assumptions and equations involved in this kind of calculation are summarized conveniently by Weiss *et al.* and will not be included here. The semiempirical parameters required for these calculations are the atomic valence state ionization potentials, W_i, the resonance integrals, β_{ij}, and the Coulomb repulsion integrals, $(ii|jj) = \gamma_{ij}$. The values chosen for these parameters are the "standard" set described by Weiss *et al.* (1965). Specifically $W_c = -11.24$ eV; $W_N = -26.64$ eV; $(ii|ii)_c = 9.49$ eV; $(ii|ii)_N = 12.12$ eV, and $\beta_{ij} = (-2.371 \text{ eV}) \cdot S(R_{ij})/S(1.39)$, where $S(R)$ is the overlap of two Slater p_z orbitals separated by a distance R (in Å). The overlap of the

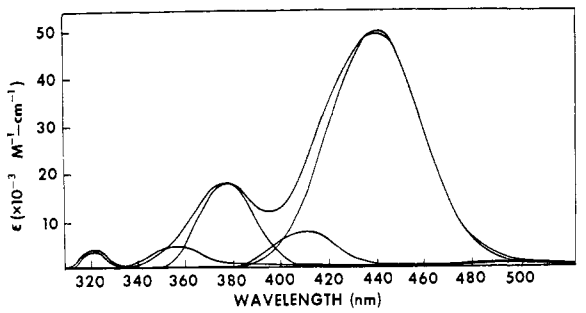

FIGURE 3: Absorption spectrum of liver alchol dehydrogenase-bound auramine O resolved into five Gaussian bands.

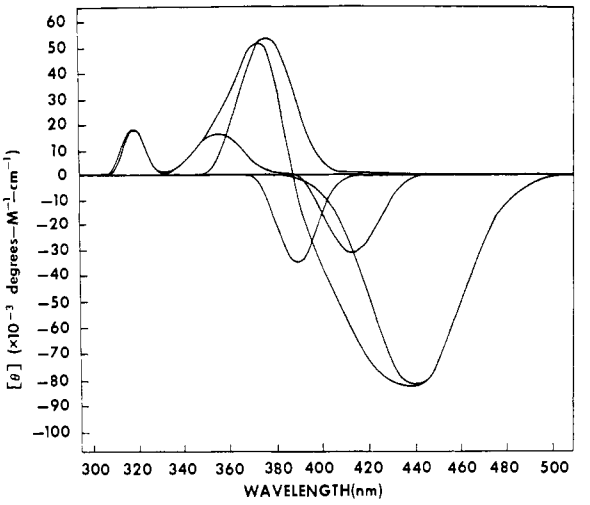

FIGURE 4: CD spectrum of liver alcohol dehydrogenase-bound auramine O resolved into six Gaussian bands.

bridge carbon orbital with those on the adjoining ring carbons depends upon the particular angle of ring rotation assumed. In these cases the overlap integrals were multiplied by the cosine of the twist angle.

The two-center repulsion integrals were calculated according to the formula of Mataga and Nishimoto (1957)

$$(ii|jj) = 14.4 \text{ eV}/(a_{ij} + R_{ij})$$

where $a_{NN} = 1.19$, $a_{CC} = 1.52$, and $a_{NC} = 1.35$. No adjustments were made for the variation in repulsion integrals due to departure from parallelism of the p_y orbitals in the various skewed conformations.

The molecular skeleton used for these calculations included only the atoms contributing to the π-electron system. No attempt was made to include the inductive effects due to the methyl substitution at the amino nitrogens in these calculations, but an empirical correction has been applied (see Results) to compensate for these neglected factors.

The various electronic transitions, their energies, and polarizations were obtained by applying configuration interaction to the relevant excited configurations yielded by the PPPSCF calculations. The mixing of 20 such configurations was considered in obtaining the final wave functions, representing all the possible transitions between the five highest energy filled molecular orbitals and the four lowest energy empty molecular orbitals.

The oscillator and rotational strengths of the electronic transitions were calculated using the dipole velocity method (Moscowitz, 1957). The matrix elements of the gradient operator were evaluated using the expressions given by Imamura *et al.* (1972).

Results

CD Spectra. The near-ultraviolet portion of the CD spectrum generated by the auramine O–liver alcohol dehydrogenase complexes consists of bands due to the extrinsic Cotton effects arising from the electronic transitions of the dye. The CD spectrum of the binary auramine O–liver alcohol dehydrogenase complex is depicted in Figure 2, along with the corresponding absorption spectrum. In Figures 3 and 4, these spectra are shown resolved into a minimum number of Gaussian bands, consistent with the constraints mentioned earlier. The oscillator and rotational strengths of these bands are listed in Table I. It is evident that all the electronic transitions appearing in the absorption spectrum were rendered optically active by the binding of the dye to the protein. The Gaussian decomposition of the CD spectra also indicated the occurrence of a negative band centered at about 388 nm, for which there is no corresponding absorption band. This band may be an artifact of the Gaussian approximation, since there is no definite theoretical evidence for its occurrence either (see MO Calculations).

The results of a similar Gaussian analysis of the data on the various ternary complexes are given in Table II. It is difficult to make precise estimates of the experimental errors in the rotational strengths because of the uncertainties inherent in the analysis into Gaussian bands. However, it is apparent from Table II that the CD spectra of the ternary complexes involving AMP, ADP, and ADPRib show relatively small and unsystematic variations in the rotational strengths compared with those of the binary complex. The largest of these variations are associated with the weaker bands, while the most intense bands show very small differences. These variations, which are at most 10–15%, are almost certainly ascribable in large part to experimental error and to shortcomings of the Gaussian analysis. Thus our study does not indicate any significant effects on the induced CD of the auramine O–liver alcohol dehydrogenase complex on binding the various co-

TABLE I: Oscillator Strengths and Rotational Strengths of the Auramine O–Liver Alcohol Dehydrogenase Electronic Transitions.[a]

λ_a[a] (nm)	f[b]	R[c] (DBM)
298[d]		*ca.* −0.12
318	0.0146	0.12
353	0.0371	0.13
376	0.186	0.68
(388)	0	−0.27
410 (414)[e]	0.064	−0.24
440	0.533	−1.44

[a] λ_a = wavelength of band center. [b] f = oscillator strength. [c] R = rotational strength. [d] This CD band was not resolved, but appeared to have approximately the same magnitude as the 318-nm band. [e] The number in parenthesis is the wavelength of the CD maximum while the other number refers to the maximum in absorption.

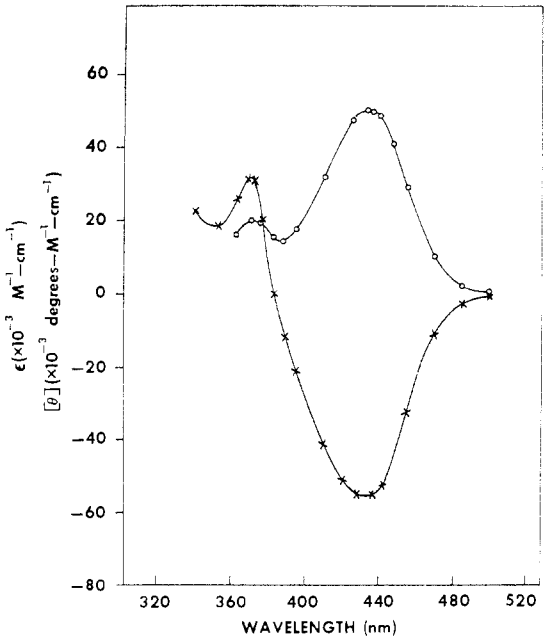

FIGURE 5: Absorption spectrum (O) and CD spectrum (X) of auramine O in ternary auramine O–liver alcohol dehydrogenase–NAD complex. Absorption spectrum: 1.6×10^{-6} M auramine O, 4.82×10^{-5} M liver alcohol dehydrogenase and 8.2×10^{-4} M NAD^+. CD spectrum: 4.83×10^{-5} M auramine O, 2.24×10^{-5} M liver alcohol dehydrogenase and 5.9×10^{-3} M NAD^+.

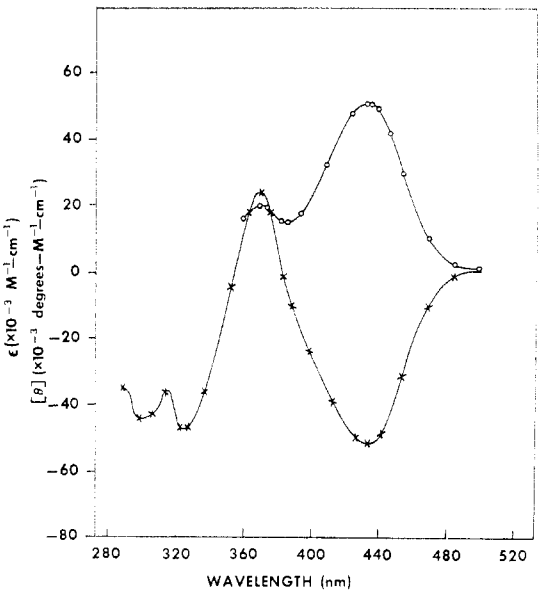

FIGURE 6: Absorption spectrum (O) and CD spectrum (X) of auramine O in ternary auramine O–liver alcohol dehydrogenase–NADH complex. Absorption spectrum: 1.6×10^{-6} M auramine O, 4.82×10^{-5} M liver alcohol dehydrogenase and 1.00×10^{-4} M NADH. CD spectrum: 4.76×10^{-5} M auramine O, 2.30×10^{-5} M liver alcohol dehydrogenase and 6.3×10^{-5} M NADH.

enzyme fragments. This result is consistent with the fluorescence studies of Heitz and Brand (1971) who found no fluorescence intensity changes on forming ternary complexes with AMP, ADP, and ADPRib, after corrections for enhanced auramine O binding were made. The spectra of the ternary complexes with NAD^+ and NADH are shown in Figures 5 and 6, respectively. In each case the strong bands centered at 440 and 376 nm undergo blue shifts of approximately 5 nm. Also, the rotational strengths of these transitions are diminished significantly with respect to the values obtained for the binary complex.

Molecular Orbital Calculations. To arrive at an understanding of the electronic transitions involved in the CD spectra of the various auramine O–liver alcohol dehydrogenase complexes, and to test the suitability of the PPPSCF approach, the molecular orbital calculations were initially carried out on the less complicated molecule, the dimethylaminobenzaldiminium ion (II). This compound can be regarded essentially as the monomeric unit of which auramine is the dimer. We expected that an analysis would indicate the existence of a low energy charge-transfer transition in the vicinity of 400 nm, which is the approximate midpoint in energy of the strong transitions exhibited by the auramine spectrum. This CT transition corresponds to the transfer of an

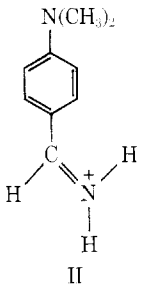

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TABLE II: The Effects of Ternary Complex Formation on the Rotational Strengths of the Auramine O–Liver Alcohol Dehydrogenase CD Bands.

	Additions					
λ_e (nm)	*R* (DBM)	+ AMP *R* (DBM)	+ ADP *R* (DBM)	+ ADPR *R* (DBM)	+ NAD *R* (DBM)[a]	+ NADH *R* (DBM)[a]
318	0.12	0.12	0.12	0.13		
353	0.13	0.12	0.19	0.16		
376	0.68	0.74	0.80	0.68	0.41 (371)	0.43 (371)
388	−0.27	−0.20	−0.36	−0.30	−0.08	−0.32
414	−0.24	−0.30	−0.21	−0.24	−0.21	−0.16
440	−1.44	−1.44	−1.51	−1.50	−0.89 (435)	−0.87 (435)

[a] Parenthesized values indicate wavelengths of the CD bands which undergo shifts from their positions in the binary spectrum.

TABLE III: Electronic Transitions of Dimethylaminobenzaldiminium Ion Obtained from Molecular Orbital Calculations.

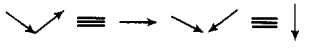
λ_a (nm)	Polarization[a]	f
178	z	0.289
211	x	0.202
342	z	0.328
423	x	0.013

[a] The z axis is the long axis of the molecule and the x axis is the short in-plane axis.

electron from the dimethylamino group (a good donor) to the protonated imino group (a good acceptor).

Four electronic transitions were found to occur in the region of spectral interest for the dimethylbenzaldiminium ion; these are listed in Table III, along with the calculated polarizations and oscillator strengths.[2] The strong band centered at 342 nm is polarized along the long axis of the molecule and can be identified as the CT transition. The very weak band at 423 nm is analogous to the "forbidden" L_b band (Klevens and Platt, 1949) of benzene. The two higher energy bands correspond to B-type transitions in the benzene ring. The spectral location of the CT band is brought into better accord with expectations by correcting for the inductive effect of the two methyl substituents on the amino group, which was not considered in the calculations. By decreasing the ionization potential of the amino nitrogen, the dimethyl substitution would lower the energy of the CT transition to an extent corresponding to about 27 nm (Scott, 1964) in its spectral location. Thus the theoretical CT band of dimethylaminobenzaldiminium occurs at 369 nm.

In auramine the CT transitions can involve both rings. One might expect these transitions to interact in symmetric and antisymmetric fashions, yielding two CT bands which are split by their interaction energy and which have different polarizations, as indicated below.



The symmetric interaction on the right should lead to a higher energy CT band polarized along the axis of C_2 symmetry (the z axis); the antisymmetric interaction should lead to a lower energy band polarized along the long molecular axis (the x axis). The approximately 120° angle through which these transitions interact suggests that the ratio of the intensities of the long- and short-wavelength components should be approximately 3:1. This is in accord with the measured values of the oscillator strengths of these bands (see Table I) and with the finding (Adam, 1960) that these are the polarizations of the experimental bands.

This intuitive assignment was verified by molecular orbital calculations on six different models of auramine, each defined by a particular angle of conrotatory twist of the phenyl rings around the bonds to the imino carbon.

The results of the configuration interaction calculations indicated that there were seven electronic transitions in the relevant spectral range. The locations, oscillator strengths, and rotational strengths of these transitions were found to depend on the twist angles, but their polarizations remained

[2] See paragraph at end of paper regarding supplementary material.

TABLE IV: Electronic Transitions of Auramine O obtained from Molecular Orbital Calculations on 45° Model.

λ_a (nm)	Polarization[a]	Assignment
481	xy	CT
434	xy	L_b
434	z	L_b
347	z	CT
235	xy	B_b
235	z	B_b
232	xy	B_a

[a] The x axis is the long axis of the molecule and the z axis is the axis of C_2 symmetry.

the same. These bands and their characteristic polarizations are listed in Table IV along with the assignments for the 45° model. The two general kinds of transitions were (a) those involving an oscillation of charge through the entire molecule and (b) those involving local excitations of the benzene rings. Bands of the former type were calculated to be at 481 and 347 nm for the 45° model. The long-wavelength band involves transfer of charge between the two dimethylaminophenyl chromophores *via* the imino group and is polarized along the axis of the molecule. The shorter wavelength CT band involves charge oscillation from these chromophores to the imino group, and is accordingly polarized along the symmetry axis of the molecule. These bands can be readily identified with the experimental bands at 440 and 376 nm.

At 434 nm two bands were found, corresponding to symmetric and antisymmetric combinations of the L_b bands mentioned in connection with the transitions in dimethylaminobenzaldiminium ion. The weakness of these bands accounts for the lack of splitting arising from this coupling. Together, they can be identified with the 410-nm experimental band.

Finally, three transitions were identified at shorter wavelengths. Two of these occur at 235 nm and are associated with symmetric and antisymmetric combinations of the B_b benzene bands, in which charge oscillates laterally across the benzene rings. The band at 232 nm is a B_a transition. Although rather higher in energy than the experimental bands observed at 318 and 298 nm, these transitions have certain features that suggest their identification with these bands, as will be considered shortly.

The spectral location, oscillator strengths, and rotational strengths of these bands are listed in Tables V and VI for each of the twist angles used in the calculations. The experimental values are also included for comparison. The wavelengths of the CT and L_b transitions are seen to increase steadily with increasing values of the twist angle, while those of the B bands are insensitive to such changes. Both the CT and L_b, bands involve the promotion of an electron to a molecular orbital which has a high antibonding electron density on the imino group, and which also exhibits nodes between the bridge carbon and each of the benzene rings. This antibonding overlap decreases with increasing twist angles so that the energy of the orbital assumes more negative values. Therefore all the transitions involving this orbital are shifted to lower energies as the twist angle is increased. The oscillator strengths of the CT and L_b transitions also depend upon the overlap of the ring π-electron systems with the p_y orbital on the imino carbon. The values calculated for this function are seen to de-

TABLE V: Spectral Location and Oscillator Strengths of the Calculated Electronic Transitions of Auramine O as a Function of the Twist Angle.

	CT_x[a]		CT_z[a]		L_b[b]		B_b[b]		B_a	
θ (deg)	λ_a (nm)	f	λ_a (nm)	f	λ_a (nm)	f	λ_a (nm)	f	λ_a (nm)	f
0	423	0.515	308	0.081	398	0.042	233	0.130	226	0.037
20	439	0.498	312	0.079	401	0.035	233	0.100	224	0.043
30	450	0.475	322	0.078	409	0.027	233	0.101	226	0.033
45	481	0.402	347	0.072	434	0.015	234	0.095	231	0.022
60	544	0.277	404	0.054	489	0.005	236	0.105	244	0.002
90	1114	0	1114	0	810	0	300	0	300	0
Experiment	441	0.533	376	0.186	410	0.064	318	0.025	298	

[a] CT_x and CT_z refer to the x- and z-polarized CT transitions, respectively. [b] These bands consist of two electronic transitions.

crease substantially with increasing twist angles, ultimately becoming zero as the overlap vanishes in the 90° model.

The calculated values of the rotational strengths of these transitions are generally more sensitive functions of twist angle than are wavelength and oscillator strength, and are therefore most useful in selecting among the possible conformations of enzyme-bound auramine. The signs of the rotational strengths are also uniquely able to provide information on the sense of the twist angles as well. The values listed in Table VI relate to a left-handed twist angle. For right-handed angles their signs would be reversed.

Of the two CT transitions the long-wavelength, x-polarized transition has by far the greater intrinsic rotational strength. It is evident that this should be so, since charge is both displaced along, and rotated around the x axis as it oscillates between the rings. Thus, the transition has a magnetic moment parallel to the electric dipole moment. The magnetic moment increases with increasing twist angle, so that the rotational strength has a strong angular dependency. A rotational displacement of charge also accompanies the z-polarized CT transition, so that the same angular dependency of the rotational strength is observed for this transition, although its absolute value is much smaller.

The transitions resulting from local excitations in the benzene rings are also optically active when the rings are twisted from coplanarity. That is, the individual L_b (or B_b) transitions can combine in both symmetric and antisymmetric fashions. Since they are skewed, they behave to a first approximation as coupled oscillators (Schellman, 1968), and generate CD bands that are equal in magnitude but opposite in sign. Since some coupling also occurs with other transitions, the oppositely signed components are not of precisely the same magnitudes, so that a net Cotton effect remains associated with these transitions. In Table VI only the resultant rotational strengths are shown.

TABLE VI: The Variation of Calculated Rotational Strength as a Function of Twist Angle.

θ (deg)	R_{CT_x} (DBM)	R_{CT_z} (DBM)	R_{L_b} (DBM)[a]	R_{B_b} (DBM)[a]	R_{B_a} (DBM)
0	0	0	0	0	0
20	−1.09	−0.17	0.05	−0.09	−0.06
30	−1.47	−0.24	0.12	−0.10	−0.09
45	−1.86	−0.34	−0.15	0.06	−0.14
60	−2.02	−0.40	0.13	0.12	−0.07
90	0	0	0	0	0
Experiment	−1.44	0.68	−0.24	0.12	~−0.12

[a] These rotational strengths are the sums of two oppositely signed contributions.

Discussion

The electronic transitions obtained from the MO calculations account quite satisfactorily for the experimental absorption spectrum and for most of the features of the CD spectra. Two bands that did not appear in the calculations, however, were the relatively insignificant ones centered at 353 and 388 nm. The latter, in fact, was present only in the CD spectrum, so that it is very likely an artifact induced by the multiplicity of CD bands on which the Gaussian approximation had been imposed.

The theoretical transitions in the region between 225 and 235 nm are probably associated with the experimental pair of oppositely signed CD bands at 318 and 298 nm. Even though they occur at somewhat higher energies than the experimental bands, their oscillator and rotational strengths agree quite favorably.

The rotational strengths of the composite L_b bands and B_b bands depend in both sign and magnitude on the twist angle, as can be seen from Table VI. These values agree most favorably with those of the experimental spectra for the 45° model. At this twist angle the theoretical and experimental CD bands are in good agreement with the exception of the short-wavelength CT transition. The calculations indicate this transition to have a small, negative intrinsic rotational strength instead of the substantial positive value actually observed. This discrepancy is unfortunate, but not crucial, since the oscillator strength of this transition is sufficient for it to couple effectively with transitions originating in the protein chromophores, and to derive its observed positive rotational strength from this coupling.

The sign and magnitude of the Cotton effect associated with the long-wavelength CT transition provide the principal means for concluding that the sense of the twist is left-handed. According to the rule enunciated by Hug and Wagnière (1972) for chromophores of C_2 symmetry, the long-wavelength CT transition, which is of symmetry B, should give rise to a negative Cotton effect if the sense of twist is left-handed. The close correspondence of the calculated values of the intrinsic rotational strength for this transition based on left-

handed models with its experimental value leads us to conclude that the auramine is bound as a left-handed conformer.

Although the sense of the twist is clearly established by the calculations, the exact angle is much more difficult to specify. The most probable value appears to be between 20 and 45°, as indicated by a comparison of theoretical and experimental results in Tables V and VI. The signs of the Cotton effects associated the L_b and B_b transitions are in agreement with experiment only for angles close to 45°. Since these are each attributable to the sum of large, oppositely signed components, however, there is expected to be considerable uncertainty in their calculation. The magnitude of the large negative CD band associated with the long-wavelength CT transition is best reproduced for a twist angle of 30°. Because this transition has a large oscillator strength, however, it may acquire a significant portion of its rotational strength by coupling with protein transitions, so that the intrinsic contribution to the observed value of −1.43 DBM is uncertain. As the angle of twist exceeds 30°, the intrinsic rotational strength of the 376-nm band becomes more *negative*. Therefore, for angles in excess of about 30°, it becomes increasingly difficult to rationalize the observed *positive* sign of this band, in that very strong coupling with protein chromophores must be invoked. Since the oscillator strengths of the CT transitions diminish for twist angles above 45°, this required coupling seems particularly unlikely.

Other considerations favor a twist angle of about 30°. The theoretical oscillator strengths for the CT and L_b bands are in best agreement with the experimental values for twist angles between 0 and 30°. The spectral location of these bands would also seem to favor a twist angle of about 30°, although this is by no means solid evidence, considering the approximations involved in the PPPSCF calculations. That is, the position of the L_b bands agrees best with experiment at 30°. The energetic midpoint of the CT bands occurs at 405 nm. When the inductive effect of the dimethylamino group is added in, the energetic midpoints obtained from the 30 and 45° models are respectively 402 and 430 nm. Finally, the X-ray structure (Lobanova, 1968) of benzophenone, a close analog of auramine O, shows a twist angle of 30°.

Any attempts to pinpoint this angle more precisely, however, would not be justified by the theoretical methods employed, and it must suffice to say that the twist angle is left-handed, and probably no greater than 45° in magnitude.

Changes occurring in the experimental CD bands of liver alcohol dehydrogenase-bound auramine in response to the binding of other ligands to the enzyme can originate from alterations in either the intrinsic rotational strengths of these bands, or the extent to which they couple with transitions in the protein chromophores. Changes of the former kind would be most readily interpreted as indicating a change in the twist angles of the bound auramine, whereas those due to the latter source would indicate some alteration in the asymmetric environment of the dye. Either of these types of changes may occur as a result of conformational changes on binding the coenzyme or coenzyme fragments or as a result of direct interaction with the additional ligand.

As shown in Table II, no significant changes in the CD spectrum accompany the binding of any of the coenzyme fragments. Thus, there is no evidence from CD of any conformational changes accompanying the binding of coenzyme fragments. The enhancement of the auramine O binding constant which results from ternary complex formation with coenzyme fragments (Heitz and Brand, 1971; Sigman and Glazer, 1972) may represent evidence for such conformational changes. However, this enhancement could also be attributable to the favorable electrostatic interaction of the cationic dye and anionic coenzyme fragments.

The binding of either NAD^+ or NADH to the enzyme is accompanied by a large reduction in the rotational strength of both CT bands and by blue shifts in their absorption spectra of about 5 nm. The direction of these changes is consistent with a decrease in the twist angle of the bound dye. However, it would appear that the large changes in the rotational strengths of the CT transitions cannot be solely attributed to changes in the conformation of the bound dye. From Table V we see that a blue shift of about 5 nm would correspond to a change in θ of only 2–3°. This would not suffice (see Table VI) to account for the nearly twofold change in the rotational strengths of the CT transition. Thus we must attribute at least part of this change to differences in the coupling of the CT transition to other groups.

Are these changes in coupling due to conformational changes in the protein on binding the coenzymes or are they due to direct interaction of the dye and the coenzyme? Although we cannot entirely rule out the latter possibility, we strongly favor the former explanation for two reasons. First, the observed changes are very similar for both NAD^+ and NADH. If the auramine O transitions were coupling significantly with the nicotinamide transitions, one would expect rather different effects from the oxidized and reduced forms in view of their very different spectral properties and the postulated (Heitz and Brand, 1971) differences in position of the oxidized and reduced nicotinamide moiety. Second, in the ternary complex with NADH, we can observe the CD band at *ca.* 330 nm characteristic of the reduced nicotinamide moiety. The CD in this region (not shown here) is a linear combination of the CD of the two binary complexes. Such a result would not be expected if coupling of auramine O and NADH transitions were significant.

Thus, the CD spectra of the ternary complexes involving liver alcohol dehydrogenase, auramine O, and intact coenzymes provide evidence for the existence of a conformational change in the dehydrogenase on binding NAD^+ or NADH and indicate that the presence of the nicotanamide moiety is essential. This result is consistent with the observations of Heitz and Brand (1971) on fluorescence quenching of liver alcohol dehydrogenase bound auramine O which is exhibited only in ternary complexes with intact coenzyme and not with coenzyme fragments. In addition, there is evidence from X-ray diffraction studies (Zeppezauer *et al.*, 1967) that a conformational change occurs in the dehydrogenase upon binding either NAD^+ and NADH, but not upon binding coenzyme fragments.

Finally, this study suggests that the auramine O-binding site of liver alcohol dehydrogenase has a particular geometry capable of accommodating the twisted ring system of auramine O. Our CD data and calculations indicate that only the enantiomer with a left-handed chirality binds to the enzyme. Combining this result with the picture of the active site region derived by Heitz and Brand (1971), we conclude that that the hydrophobic pocket postulated to accommodate the phenyl groups of auramine O and also larger substrates has two slots which are twisted in a left-handed sense through an angle of 40–90°.

Our results also suggest an explanation for why liver alcohol dehydrogenase is unique among all of the proteins (including several other NAD-dependent dehydrogenases) which Conrad *et al.* (1970) tested in binding auramine O so strongly. It is probable that only liver alcohol dehydrogenase has the par-

ticular twisted hydrophobic pocket in the proper relationship to a site favorable for binding the cationic iminium group.

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Supplementary Material Available

Supplementary material describing the results of the molecular orbital calculations in more detail will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20 X reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-73-3459.

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Growth, Isolation, and Characterization of a Yeast Manganese Alcohol Dehydrogenase†

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ABSTRACT: In order to elucidate the role of zinc in yeast alcohol dehydrogenase, zinc was replaced by manganese, and properties of the manganese enzyme were compared with the zinc enzyme. A wild type *Saccharomyces cerevisae* (YU 1001) was anaerobically grown in a zinc-free, manganese-rich medium as well as in a normal zinc-containing medium. The Mn enzyme was isolated and estimated to be greater than 50% homogeneous. It was less stable than the corresponding Zn enzyme, and a modified isolation and purification scheme was developed. It was necessary to eliminate all heat steps from the purification and EDTA from the buffers and to add sucrose and mercaptoethanol. Both the Mn and the Zn enzymes from YU 1001 interact with antibodies prepared against baker's yeast alcohol dehydrogenase suggesting that their structures were similar to each other as well as to the baker's enzyme. The K_m values for the two YU 1001 enzymes were similar and close to the values for the baker's yeast enzyme; however, their R_F values on gel electrophoresis as well as their heat stabilities and ammonium sulfate solubilities differed. The pH profile for the three enzymes also differs such that the YU 1001 enzymes have a narrow bell-shaped curve with pK_{app} values of 6 and 8.5 for the Zn enzyme and 7 and 8.5 for the Mn enzyme. It could not be ascertained whether the two YU 1001 enzymes were products of the same gene or whether the presence of manganese altered the structure of the protein accounting for the lower stability of the Mn enzyme.

Alcohol dehydrogenases characterized from many different sources are invariably zinc metalloenzymes (references in Sund and Theorell, 1963). For this reason the role of zinc in the enzyme, especially that isolated from horse liver, has been extensively studied. The zinc-free horse liver enzyme is catalytically inactive (Åkeson, 1964; Oppenheimer *et al.*, 1967); however, it binds coenzyme and coenzyme analogs (Weiner, 1969; Mildvan and Weiner, 1969; Hoagstrom *et al.*, 1969) and substrate and substrate analogs (Iweibo and Weiner, 1972; Coleman *et al.*, 1972a), and retains its native molecular weight (Green and McKay, 1969; Coleman *et al.*, 1972a). In addition, the lack of catalysis with an activated substrate, bromoacet-

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