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A Computerized Calibration of the Circular Dichrometer*

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ABSTRACT: A least-squares parametric curve-fitting method is used to calibrate a circular dichrometer against a standardized spectropolarimeter. An application of this technique is demonstrated by determining the "calibrated" molar circular dichoic absorption, $\epsilon_l - \epsilon_r$, of two camphor derivatives in solution. Maximal values of +2.20 for *d*-10-camphorsulfonic acid in water, +1.54 for *d*-camphor in methanol, and +1.69 for *d*-camphor in dioxane are found at 291, 296, and 300 mμ, respectively. It is concluded that these values may be used as standards for calibration provided some precautions regarding solute and solvent purity are taken.

Circular dichroism is readily becoming a popular technique for conformational studies of biopolymers in solution (see, for example, Beychok, 1968; Yang, 1969; Yang and Samejima, 1969). It complements optical rotatory dispersion which has virtually monopolized the literature over the last decade. Several commercial circular dichrometers are now capable of high-precision measurements over a wide region of the spectrum. However, there is still uncertainty in the calibration of these instruments because of the lack of a universally acceptable standard. In contrast, spectropolarimeters can be calibrated with standard sucrose solutions or with quartz control plates of known rotations. In this communication we present a method of calibration based on a computer program developed by Thiéry (1968). In essence the method consists of calibrating the circular dichrometer against a calibrated spectropolarimeter.

Theory

Circular dichroism and optical rotatory dispersion are closely knit phenomena whose interdependency is contained in the Kronig-Kramers relations (Moffitt and Moscowitz, 1959; Moscowitz, 1962; Emeis *et al.*, 1967). Therefore, theoretically it is possible to effect a circular dichroism to optical rotatory dispersion transformation through an evaluation of

$$[M(\lambda)] = (2/\pi)\int_0^\infty [\Theta(\lambda')][\lambda'/(\lambda^2 - \lambda'^2)]d\lambda' \quad (1)$$

where $[M(\lambda)]$ is the molar rotation at wavelength λ, $[\Theta(\lambda')]$ is the molar ellipticity at wavelength λ', and, λ and λ' are the main variable and parameter of integration, respectively. (An analogous equation can be used to transform optical rotatory dispersion to circular dichroism.) However, eq 1 requires that the integration be carried over all the optically active bands. In practice, for obvious reasons this can never be realized. This difficulty may be circumvented by applying eq 1 over a definite domain and representing bands outside this domain with the well-known Drude equation. It is necessary that these bands be sufficiently distant from the domain to warrant the applicability of the Drude equation in the region of the domain. Under these condi-

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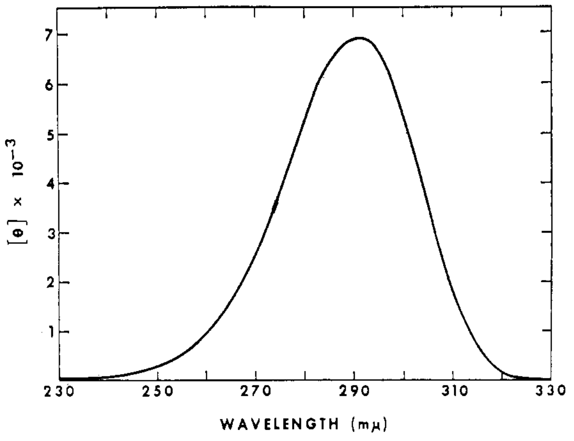

FIGURE 1: Circular dichroism of *d*-10-camphorsulfonic acid in water. The circular dichrometer is calibrated according to Durrum-Jasco standard. See text for details.

tions, the optical rotatory dispersion in a definite domain may be expressed as

$$[M(\lambda)] = (2/\pi)\int_a^b [\Theta(\lambda')][\lambda'/(\lambda^2 - \lambda'^2)]\mathrm{d}\lambda' + \sum_i a_i\lambda_i^2/(\lambda^2 - \lambda_i^2) \quad (2)$$

In applying eq 2 to experimental data, it is necessary to compensate for the uncertainty in the calibration of the dichrometer. Then eq 2 becomes

$$[M]_\lambda^{\mathrm{expt}} = f[M]_\lambda^{\mathrm{calcd}} + \sum_i a_i\lambda_i^2/(\lambda^2 - \lambda_i^2) \quad (3)$$

where $[M]_\lambda^{\mathrm{expt}}$ is the experimental optical rotatory dispersion of a compound, $[M]_\lambda^{\mathrm{calcd}}$ the calculated value from the measured circular dichroism of the same compound, and f a correction factor to compensate wavelength-independent errors in the calibration of the circular dichrometer. Our criterion of calibration is based on the value of this f factor relative to an absolutely calibrated spectropolarimeter. Calibration is assumed to be achieved under those conditions which make this factor f unity. For circular dichrometers using an electrooptic polarization modulator (for example, Cary, Durrum-Jasco, and Roussel-Jouan instruments), this can be realized by adjusting the ac gain of the variable amplifier to an appropriate value determined by the solution of eq 3. Alternatively, one can determine the f factor for a particular setting of the ac gain and simply multiply all experimental data by this value.

Experimental Section

Materials. *d*-10-Camphorsulfonic acid and *d*-camphor were reagent grade (Eastman Kodak). *d*-10-Camphorsulfonic acid was used without further purification. *d*-Camphor was purified by repeated sublimations. Methanol and dioxane were of spectroquality (Matheson Coleman and Bell) and were used as obtained. Double-distilled water was used for aqueous solutions. Sucrose was of the National Bureau of Standards grade. Solutions of *d*-10-camphorsulfonic acid and *d*-camphor were prepared volumetrically to a solute concentration of about 0.2%.

Methods. Circular dichroism was measured with a Jasco UV–ORD-5 spectropolarimeter with a circular dichroism attachment and optical rotatory dispersion with a Cary 60 spectropolarimeter. Specially designed thermostatable cell holders and jackets were installed in both instruments. Temperature was maintained at 25.0 ± 0.1° by a Haake constant-temperature circulator. Both circular dichroism and optical rotatory dispersion of a compound were measured with the same solution. Circular dichroism spectra were recorded with a 1.00-cm path length on the ± 0.02 difference in absorption full-scale range of the Jasco and a scan speed of 0.12 Å/sec. Optical rotatory dispersion spectra were recorded with a 2.00-cm path length on the 1.0° full-scale range of the Cary and a scan speed of 0.1 Å/sec. Slit widths of both instruments were maintained for optimum spectral purity and energy throughout the spectral region.

The spectropolarimeter was calibrated with a freshly prepared sucrose solution. The results were in excellent agreement with those of Lowry (1935) over the 27 wavelengths compared, as judged by an average absolute per cent deviation of 0.4. This is compatible with the expected reliability of the data points.

Programs were written on FORTRAN IV for CDC 6400 and for IBM 7094 (Thiéry, 1968). Computations were carried out at the Computing Center of the University of California at Berkeley. Circular dichroism and optical rotatory dispersion were expressed in terms of molar circular dichroic absorption, $\epsilon_l - \epsilon_r$, and molar rotation, [M], respectively.

Results and Calculations

To illustrate our method of calibration, the circular dichroism and optical rotatory dispersion of *d*-10-camphorsulfonic acid in water were measured in the ultraviolet region (Figures 1 and 2). An isolated non-Gaussian positive circular dichroism band is observed with a maximum at 290.5 mμ (Figure 1). In the shorter wavelength region another band was detected with a negative maximum centered at 192.0 mμ (not shown in Figure 1). The numerical values shown here were based on a calibration standard recommended by the manufacturer (Durrum-Jasco), which consisted of adjusting the ac gain to give an $\epsilon_l - \epsilon_r$ of +2.09 for *d*-10-camphorsulfonic acid in water at 290.5 mμ.

Before the f factor can be determined from eq 3, $[M]_\lambda^{\mathrm{calcd}}$ must first be computed from eq 1. To avoid any uncertainties in extrapolation below 185 mμ, only the positive circular dichroism band (shown in Figure 1) was considered (the fact that this band may or may not be a single band will not alter our calculation). Since it is separated by 98.5 mμ from the negative band at shorter wavelengths, the requirements for the Drude equation for eq 3 are met. Numerical integration was carried over 181 data points at 0.5-mμ intervals to minimize integration errors. The dispersive effects of the refractive index of the solvent were neglected, since in the

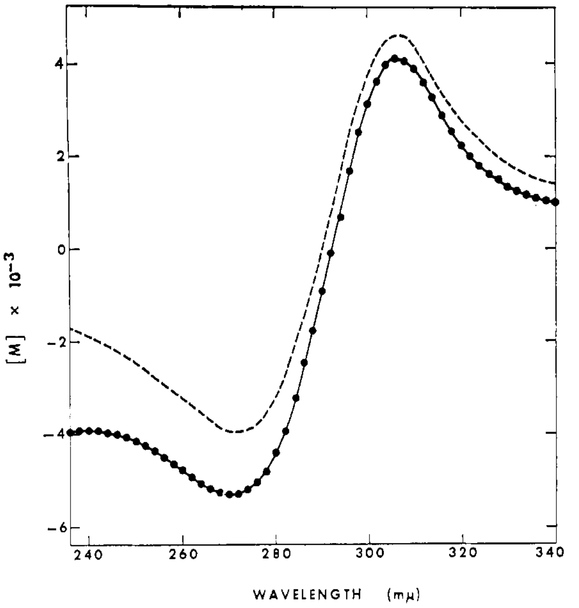

FIGURE 2: Optical rotatory dispersion of *d*-10-camphorsulfonic acid in water. Solid line: experimental; dotted line: computed from circular dichroism shown in Figure 1 using eq 1; points: computed from eq 3. See text for details.

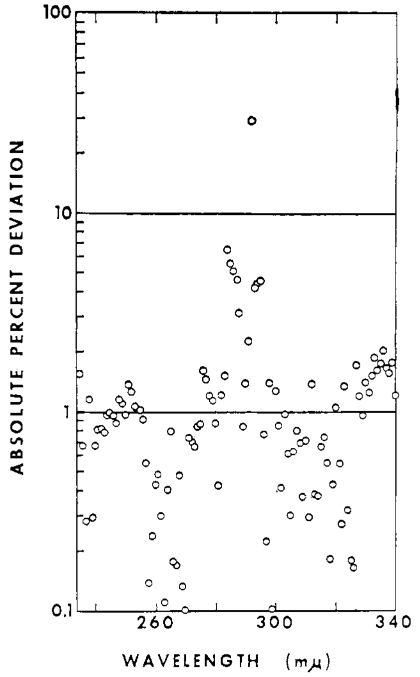

FIGURE 3: Distribution of absolute per cent deviation of calculated optical rotatory dispersion, based on eq 3, from experimental optical rotatory dispersion.

spectral region considered here they are negligible in aqueous solutions (Thiéry, 1968). The computed optical rotatory dispersion values (dotted line) corresponding to the positive 290.5-mμ circular dichroism band are compared with the experimental ones (solid line) in Figure 2. Although the deviations can largely be attributed to neglect of other optically active bands in the computation of $[M]_\lambda^{\text{calcd}}$ (background rotation), they may partially be due to uncertainty in the calibration of the circular dichrometer.

The "best-fit" values of the f factor and the Drude parameters were obtained from eq 3 by a least-square curve-fitting program. The computer was provided with initial guesses for f and the Drude parameters along with calculated and experimental optical rotatory dispersion and wavelengths. The initial guesses were modified until the computed rotations on the right-hand side of eq 3 were matched to the experimental ones on the left-hand side. The program was terminated when the sum of the squares converged to relative change of 10^{-5}. A single Drude term was sufficient in all cases for convergence. The addition of more terms in eq 3 did not increase the precision of "fit" and in some cases prevented convergence. The "best-fit" values of the computed optical rotatory dispersion (shown in Figure 2 as full circles) were obtained from an f value of 1.055 for the instrument calibrated in the Durrum-Jasco manner. The "goodness of fit" as exemplified by the absolute per cent deviation of computed rotations from experimental ones is illustrated in Figure 3 for a typical run at various wavelengths. The deviations cluster about the 1% value except near the crossover (zero rotation) where $d[M]_\lambda/d\lambda$ is very large and $[M]_\lambda$ is small. The average absolute per cent deviation determined over the entire wavelength region studied (235–340 mμ) in increments of 0.5 mμ was 1.61 which approaches the uncertainty in the data points.

It is apparent that the f factor is not a constant of the instrument but varies with the arbitrary setting of the instrument ac gain. However, the product of the f factor and the $\epsilon_l - \epsilon_r$ value obtained from any setting of the ac gain should be constant. This constancy was verified experimentally by repeating the above analysis based on eq 3 for *d*-10-camphorsulfonic acid at several arbitrarily chosen values of the ac gain. The values obtained were constant within an experimental uncertainty of 1%.

To verify that the f value for the same setting of the ac gain does not vary with the solute–solvent combination used, we repeated computations for *d*-camphor in methanol and dioxane and compared them with those obtained with *d*-10-camphorsulfonic acid in water (the ac gain was maintained at the same level during these experiments). The f values are in excellent agreement with a maximum variation of 0.004 (Table I). The average absolute per cent deviations are also very similar in all three cases.

The $\epsilon_l - \epsilon_r$ values listed in Table I have been corrected to an f factor of unity. Reproducibility was within 1% of the given values. Although the f factor determined from any setting of ac gain is independent of the purity of the compounds used for calibration, the value of $\epsilon_l - \epsilon_r$ is not. Therefore, for extreme accuracy it is advisable to base calibration on the direct determination of the f factor from eq 3. However, for routine calibrations our $\epsilon_l - \epsilon_r$ values can be used as standards, provided some precautions mentioned below are taken.

Table II lists the molar rotations of *d*-10-camphorsulfonic acid and *d*-camphor in solutions used for determining $\epsilon_l - \epsilon_r$. By combining these values with $(\epsilon_l - \epsilon_r)_{\max}$

TABLE I: Calibration of Circular Dichrometer with *d*-Camphor Derivatives.

Solute	Solvent	λ_{max} (mμ)	f^a	Av Absolute % Deviation	$(\epsilon_l - \epsilon_r)_{max}{}^b$	$[\Theta]_{max}{}^d$
d-10-Camphor-sulfonic acid	Water	290.5	1.055	1.61	$+2.20^c$	+7260
d-Camphor	Methanol	295.5	1.051	1.56	+1.54	+5080
d-Camphor	Dioxane	299.5	1.053	1.66	+1.69	+5570

[a] Based on the Durrum-Jasco calibration standard. [b] Corrected values with $f = 1.00$. [c] Value for pH 2.68 (variation of this value with pH from 1.3 to 11.5 was found to be insignificant). [d] Molar ellipticity is related to molar circular dichroic absorption by $(\Theta) = (2.303)(4500/\pi)(\epsilon_l - \epsilon_r)$.

TABLE II: Optical Rotation of *d*-Camphor Derivatives.

		Peak		Trough	
Solute	Solvent	λ (mμ)	$[M]$	λ (mμ)	$[M]$
d-10-Camphorsulfonic acid	Water	306	+4120	270	−5300
d-Camphor	Methanol	313	+3150	275	−3370
d-Camphor	Dioxane	316	+3980	277	−3500

the problem of sample purity or error in concentration determination can be circumvented. For instance, with *d*-10-camphorsulfonic acid in water, the *f* factor can be determined from the following relation: $f = [2.20/(\epsilon_l - \epsilon_r)_{290.5}]([M]_{306}/4120)$. A similar relationship can also be written using the trough value at 270 mμ. This procedure is, of course, valid only when the impurities are optically inactive. But by using both *d*-10-camphorsulfonic acid and *d*-camphor for calibration, the effects of optically active impurities can be detected or minimized.

Discussion

It is instructive to compare our $(\epsilon_l - \epsilon_r)_{max}$ values with those previously reported. Table I shows that our value for *d*-10-camphorsulfonic acid in water is about 5.5% higher than that recommended by the manufacturer (Durrum-Jasco). Velluz *et al.* (1965) have compiled circular dichroism extrema of many organic compounds. They reported an $\epsilon_l - \epsilon_r$ of +1.6 for camphor in dioxane at 300 mμ, whereas the computer program enabled us to obtain the second decimal accurately, *i.e.*, +1.69. Recently, the Cary 6001 circular dichroism attachment has become available. The manufacturer's manual also suggests *d*-10-camphorsulfonic acid in water as a standard for calibration. Their listed molar ellipticity of +308° for a 1.0-mg/ml aqueous solution in a 1.0-cm path-length cell at 290 mμ is equivalent to an $\epsilon_l - \epsilon_r$ of +2.17. This value is based on an absolute determination using their Cary 1401 circular dichroism attachment, whose precision is limited to two significant figures. (J. T. Y. thanks Dr. D. Urry for this information.) Urry and Pettegrew (1967) also used a Cary 1401 and reported an $(\epsilon_l - \epsilon_r)_{max}$ of +2.2. Fortunately, our second decimal for the circular dichroism maximum of *d*-10-camphorsulfonic acid happens to be zero. Thus our computerized value and Urry and Pettegrew's experimental one are in good agreement. The value listed in the Cary manual would, in our opinion, be about 1.5% lower than its true value. It is essential that in the future development of circular dichroism as an analytic tool some standards for calibration be adopted to facilitate comparison of data.

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The State of Iron in Hemerythrin. A Mössbauer Study*

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ABSTRACT: Mössbauer spectra, and some ancillary magnetic susceptibilities, have been measured for hemerythrin in its deoxy, oxy, and several met states, as well as for a series of model binuclear, oxygen-bridged, iron compounds. Isomer shifts and quadrupole splitting values for iron in the protein derivatives are comparable to those seen in the antiferromagnetically coupled iron model compounds. The Mössbauer spectra together with the susceptibility data and the known chemical nature of the methemerythrin complexes indicate that the two iron atoms in hemerythrin are in similar environments and are near enough to interact with each other.

The data also provide evidence that the bridge formulation, FeO_2Fe, for the active site of hemerythrin is correct.

Hemerythrin is the non-heme iron protein responsible for oxygen transport in representatives of four different invertebrate phyla (Ghiretti, 1962). Neither the binding of the iron to the protein, nor the binding of oxygen to the iron is well understood. In an attempt to improve our knowledge of these phenomena, we have studied the magnetic susceptibility and the Mössbauer spectra of hemerythrin.

Hemerythrin has a molecular weight of 108,000 and is composed of eight subunits (Klotz and Keresztes-Nagy, 1963) each of which contains two iron atoms and can bind 1 mole of oxygen. Since it is likely that the two iron atoms in each subunit are involved in oxygen binding, and are hence close together, the Mössbauer spectra should be easier to interpret than optical or magnetic resonance properties of the protein. In particular, it has been found recently (Johnson, 1966b) that the effects of strong magnetic fields on Mössbauer spectra provide criteria for characterizing iron in biological compounds. These criteria can be applied to hemerythrin also to determine whether the iron atoms are interacting magnetically and hence in close proximity.

The oxygen is believed to be bound in a bridging manner between the two iron atoms in a similar way to the binding in synthetic cobalt(II) binuclear oxygen carriers (Vogt *et al.*, 1963). The iron atoms have been assumed to be in the iron(II) state in deoxyhemerythrin, but in oxyhemerythrin the valence state could be formulated in a number of different ways, *e.g.*, Fe(III)-O_2^{2-}-Fe(III) (Klotz and Klotz, 1955) or Fe(II)-O_2-Fe(II) (Williams, 1955). In all these cases the iron could be in either high- or low-spin states.

The iron in the deoxy and oxy species can be oxidized to the iron(III) species, which can bind a variety of ligands (Keresztes-Nagy and Klotz, 1965) and could also be in either a high- or low-spin state.

A further complication arises in the iron(III) forms and in the high spin iron(II) form if, as may well be the case, the two iron atoms are binding one bridging ligand. (It has been demonstrated that in the case of azide and thiocyanate the ligands are bound in the ratio of 1 mole of ligand:2 moles of iron; Keresztes-Nagy and Klotz, 1965; Klapper and Klotz, 1968.) The iron–ligand–iron system could now give rise either to weak (antiferromagnetic) or strong (bonded) interactions.

Many of the above possibilities are open to investigation by Mössbauer spectroscopy, but in order to be confident of the interpretation, it was necessary to prepare and study simple compounds having iron–iron interactions. The compounds which we have prepared have been described previously (Lewis *et al.*,

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