Difference Spectropolarimetry as a Probe for Small Conformational Changes*

Benjamin J. Adkins† and Jen Tsi Yang

ABSTRACT: Difference optical rotation can be measured with a Cary 60 recording spectropolarimeter using a differential cell holder supplied by the manufacturer. The technique has the advantages of being more sensitive, rapid, and convenient than the usual direct optical rotation methods; greater precision should also be attainable in most cases. The instrument functions as a double-beam instrument by reflection of its single beam through reference solutions whose rotatory contributions are optically subtracted from sample solutions in the incident (unreflected) beam. The technique was reliable when tested with standard sucrose solutions. However, false levorotations were indicated when absorbing solutions were placed in the reflected beam. The magnitudes of the apparent levorotations were maximum at absorption maxima. In most experimental situations such artifacts can be conveniently compensated without additional labor by means of the difference method described. The technique was used to estimate the equilibrium constant for the complexing of lysozyme with an inhibitor, *N*-acetyl-D-glucosamine.

The need to measure small differences in optical rotation between solutions of different composition has stimulated a search for techniques to overcome certain problems inherent in such measurements, whether carried out by manual subtraction of directly measured rotations or, as we shall describe, by optical subtraction of the solution rotations with direct sensing of the *difference* rotation using the Cary 60 spectropolarimeter equipped with a differential cell holder. The problem of measurement of small rotational differences between two large rotations can be attenuated with methods similar to that employed by Chignell and Gratzer (1966). Their method increases the sensitivity of direct measurement of optical rotatory dispersion by canceling most of the "background" rotation with a solution of known rotation of opposite sign placed in series with the sample solution. In addition to the problem of measuring small rotational differences, and because of it, the problem of instrumentally generated rotational artifacts is particularly serious. Dirkx *et al.* (1964) have discussed some of the systematic errors frequently encountered in the direct measurement of optical rotation, their possible causes within the sample and instrument, and certain precautions to be taken toward their elimination. However, their discussion centers around a different instrument than the one we have used, and as might be expected the problems encountered vary with the instrument. Unfortunately, due to the sophistication of commercial recording spectropolarimeters, the systematic errors likely to be encountered may not be easily identified or corrected.

In the measurement of small rotational differences it would normally be desirable to maximize them by working at the highest possible concentrations consistent with avoidance of the well-known potential artifacts associated with high optical density of about two and the consequent stray light (Urnes and Doty, 1961). However, in some circumstances artifacts may be generated at substantially lower optical densities. For example, Resnik and Yamaoka (1966) observed apparent rotations with optically inactive, nonfluorescing solutes such as potassium dichromate even at optical densities as low as 0.5. Their experiments were performed with a Bendix Model 460-C spectropolarimeter, and the manufacturer subsequently modified their instrument and eliminated the artifacts.

We also encountered rotatory artifacts within absorption bands while using the difference optical rotation capability of the Cary 60 spectropolarimeter. In this report we describe a technique for measuring difference optical rotations, the nature of the artifacts associated with absorbing solutions, and the means by which these artifacts may be circumvented. The difference method substantially increases the utility of the instrument and should have considerable application in the study of small changes in optical rotation from a variety of sources, particularly in the presence of large background rotations. It may, for example, prove to be a valuable adjunct to circular dichroism for the study of small conformational changes in proteins involving the optical activity of side chains, *e.g.*, of cystine and aromatic residues.

* From the Cardiovascular Research Institute and the Department of Biochemistry, University of California San Francisco Medical Center, San Francisco, California 94122. *Received June 12, 1967.* This work was aided by grants from the U. S. Public Health Service (HE-06285, GM-10880, and K3-GM-3441).

† Present address: Palo Alto Medical Research Foundation, Palo Alto, Calif.

Experimental Procedures

Materials. Chemicals were obtained from the following sources: sucrose, National Bureau of Standards, sample 17; potassium dichromate, Mallinckrodt, AR; sodium chloride, Baker's Analyzed Reagent; NAG[1] and D-glucosamine, Mann Research Laboratories, M.A.; lysozyme, three-times recrystallized, Pentex, Inc., Lot 2X; and water, double distilled.

Stock solutions of lysozyme, NAG, and 0.1 M NaCl were usually clarified through a medium-porosity sintered-glass filter, though some visible turbidity did not affect readings noticeably. NAG solutions were allowed to stand for about 4 hr prior to use to permit the establishment of mutarotational equilibrium (Thomas, 1966); concentrations were computed directly from weights. Lysozyme concentrations were estimated spectrophotometrically using $E^{1\%}_{1\,cm}$ 26.9 at 280 mμ (Hamaguchi and Kurono, 1963). However, Bruzzesi *et al.* (1965) have reported a somewhat lower corresponding value of 25.32.

Mixtures of NAG and lysozyme of varying molar ratios were prepared by dilution of their stock solutions to desired concentration with 0.10 M NaCl. Aliquots (3 ml) of these solutions were either diluted again with 3 ml of 0.1 M NaCl or mixed to obtain the desired solution concentrations of LS, IS, and LIS. To minimize concentration errors the same pipet was used for aliquots withdrawn from any particular stock solution, and was manipulated in the same manner. Any small change in volume during mixing was neglected and assumed to be well within experimental error. About 30 min after adding IS to LS the constancy of the optical rotation with time showed that equilibrium was established. The pH of the mixtures ranged from 5.4 to 6.1. Within the range of pH 4–6 no dependence of rotation on pH was observed. pH-dependent aggregation of the protein was assumed to be small or nonexistent (Sophianopoulos and Van Holde, 1964; Bruzzesi *et al.*, 1965; Adams and Filmer, 1966).

Methods. Difference optical rotation was measured at 25 ± 1° with the Cary 60 recording spectropolarimeter (Cary instruments) using a differential cell holder supplied by the manufacturer. Figure 1 illustrates the arrangement of the optical components for measuring difference rotation. The incident polarized light (i beam) passes through one or more optical cells before intercepting a mirrored Faraday cell that modulates the plane of polarization and reflects the beam, which results in an inversion of the beam-oriented coordinate system. The angle between the i beam and reflected beam (r beam) is about 12°. The r beam then passes through another set of cells and into the photomultiplier tube.

The functioning of the Cary 60 spectropolarimeter in the measurement of difference rotation is based on the inversion of the beam-related coordinate system due to reflection of the beam. Thus the plane of polarization as it reaches solutions placed in the r beam has, in effect, been prerotated in a direction opposite to that imparted by solutions in the i beam. If nonbirefringent cells of equal light path are filled with the same nonabsorbing optically active solution, the rotation in the i beam is exactly canceled by that in the r beam, and only the base line of the instrument is measured. Similarly, the difference technique is able to detect existing small differences in rotation between solutions in the i and r beams because any large rotatory contributions they have in common are automatically canceled.

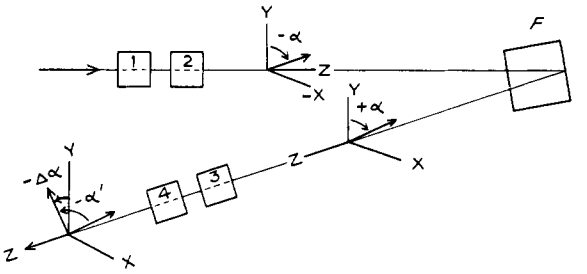

FIGURE 1: Relationship of the incident and reflected beam to the mirrored Faraday cell (F), the solution-containing cells (1–4), and the beam-oriented coordinate systems of the Cary 60 spectropolarimeter.

Experimentally, the procedure adopted was as follows. Four 1-cm cells were selected for lack of birefringence and arrayed in pairs with solutions as illustrated in Figure 1. In order to maximize beam intensity reaching each cell the solution of highest optical density in each beam was placed farthest from the light source. Then either the difference rotation or the difference optical rotatory dispersion was recorded. Ideally, nothing more than measurement of a base line would be required for the calculation of the desired difference quantity. However, this procedure was permissible only for essentially nonabsorbing solutions. Absorbing solutions placed in the r beam generated artifacts (see Results). This complication may be circumvented by performing a second measurement of the difference rotation or difference optical rotatory dispersion with cells 3 and 4 in the i beam and cells 1 and 2 in the r beam. For solutions allowing the i and r beam optical densities to be approximately equal, subtraction of one set of measured difference rotations from the other cancels essentially all the artifact; the remainder is approximately two times the actual difference rotation.

Calculation of $\Delta\alpha$. The net measured rotation of two or more solutions may be represented as the sum of contributions from the optical activity of the solutions themselves (α_j), from the optical cells (α_{cj}), and in some cases from rotational artifacts (α_{aj}), where j refers to one of the numbered optical cells. Thus the net rotation for the solution pair in cells 1 and 2, and 3 and 4, may be written as

$$\alpha_{12} = (\alpha_1 + \alpha_2 + \alpha_{c12} + \alpha_{a12}) \qquad (1)$$

[1] Abbreviations used: NAG, *N*-acetyl-D-glucosamine (a lysozyme inhibitor); S, solvent (0.10 M NaCl); LS, lysozyme plus solvent; IS, inhibitor plus solvent; and LIS, lysozyme plus inhibitor plus solvent.

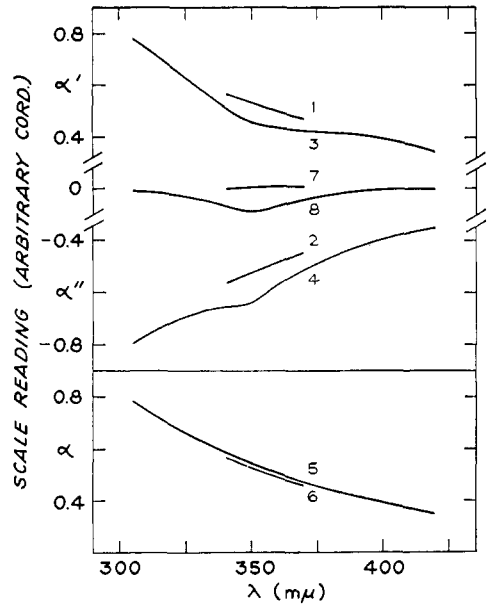

FIGURE 2: Difference rotation of sucrose in water with and without potassium dichromate solution in series. Curves: 1, sucrose *vs.* water; 2, water *vs.* sucrose; 3, sucrose plus dichromate *vs.* dichromate plus water; 4, dichromate plus water *vs.* sucrose plus dichromate; 5, calculated from 3 and 4 with eq 5; 6, calculated from 1 and 2 with eq 5; 7, base line calculated from 1 and 2 with eq 7; and 8, base line calculated from 3 and 4 with eq 7. Concentrations: sucrose, 0.101 g/dl in water, and potassium dichromate, 68.6 mg/l. in water. Cells: 1-cm path length. See text for details.

$$\alpha_{34} = (\alpha_3 + \alpha_4 + \alpha_{c34} + \alpha_{a34}) \quad (2)$$

where, for example, α_1 refers to the rotational contribution from the solution in cell 1, α_{c12} to that from optical cells 1 and 2 alone, and α_{a12} to that from apparent rotations (artifacts) generated by the solutions within cells 1 and 2. These two complementary pairs of cells may be placed in either of the two beams, giving rise to two equations for the respective net measured difference rotations $\Delta\alpha'$ and $\Delta\alpha''$

$$\Delta\alpha' = \alpha_{12} - \alpha_{34} = (\alpha_1 + \alpha_2 + \alpha_{c12} + \alpha_{a12})_i - (\alpha_3 + \alpha_4 + \alpha_{c34} + \alpha_{a34})_r + b \quad (3)$$

$$\Delta\alpha'' = \alpha_{34} - \alpha_{12} = (\alpha_3 + \alpha_4 + \alpha_{c34} + \alpha_{a34})_i - (\alpha_1 + \alpha_2 + \alpha_{c12} + \alpha_{a12})_r + b \quad (4)$$

where subscripts i and r refer to the i and r beams, b represents the instrument base line, and the minus sign accounts for the reflection and inversion of the beam-oriented coordinate system. For example, a dextrorotatory solution gives a negative reading on the recording chart when the solution is placed in the r beam instead of the i beam.

From the physical situation other useful relationships

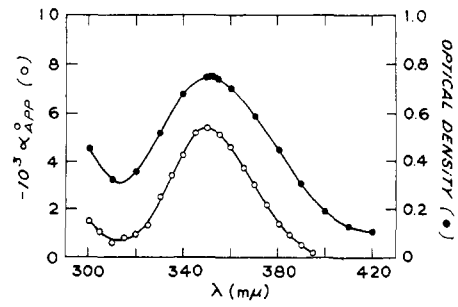

FIGURE 3: Apparent rotation at an absorption band of potassium dichromate solution (68.6 mg/l. in a 1-cm cell) placed in the reflected beam.

are apparent. Obviously, $(\alpha_j)_i = (\alpha_j)_r$. For cells with residual birefringence, $(\alpha_{cj})_i \simeq (\alpha_{cj})_r \neq 0$; these quantities must be considered only approximations because of the divergence of the light beam and because the light beam may pass through a different area of the cell face after transfer to the other beam. For cells without birefringence the $(\alpha_{cj}) = 0$ as will be assumed in the following discussion. Finally, because the rotational artifacts were produced only by absorbing solutions placed in the r beam (see Results), $(\alpha_{aj})_i = 0$ and $(\alpha_{aj})_r \neq 0$.

The true difference rotation $\Delta\alpha$ may be approximated from the difference of the two measured rotations:

$$\Delta\alpha = (\Delta\alpha' - \Delta\alpha'')/2 \quad (5)$$

Making the indicated substitutions and collecting terms yields

$$\Delta\alpha = (\alpha_1 + \alpha_2) - (\alpha_3 + \alpha_4) + (\alpha_{a12} - \alpha_{a34})_r/2 \quad (6)$$

if the instrument base line (b) is constant between measurements. For cases in which the i and r beam optical densities are approximately equal, $(\alpha_{a12})_r \simeq (\alpha_{a34})_r$, and eq 6 yields the true $\Delta\alpha$ within experimental error. For situations in which this would not be true the magnitude of the error represented by the last term of eq 6 can be evaluated by separately measuring α_{12} and α_{34} in both the i and r beams; the differences, $(\alpha_{12})_i - (\alpha_{12})_r$ and $(\alpha_{34})_i - (\alpha_{34})_r$, will yield the values for $(\alpha_{a12})_r$ and $(\alpha_{a34})_r$.

The apparent base line in the presence of artifacts, b_a, may be obtained from the average of $\Delta\alpha'$ and $\Delta\alpha''$

$$b_a = (\Delta\alpha' + \Delta\alpha'')/2 = -(\alpha_{a12} + \alpha_{a34})_r + b \quad (7)$$

Results

Test of the Method. The instrument was tested by placing nonabsorbing sucrose solution in cell 1 and water in cell 3 (Figure 1). Cells 2 and 4 were not used. The difference rotation was recorded (curve 1, Figure 2) and, after interchanging cells 1 and 3, recorded again (curve 2, Figure 2). As can be seen curves 1 and 2 are mirror images of the instrument base line (curve 7,

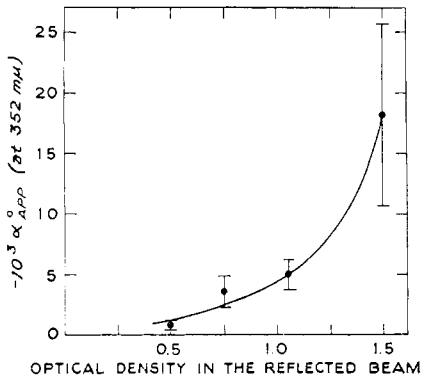

FIGURE 4: Dependence of the apparent rotation on the optical density of potassium dichromate solutions placed in the reflected beam. $K_2Cr_2O_7$ concentration: 68.6 mg/l. Path lengths: 5, 10, 15, and 20 mm.

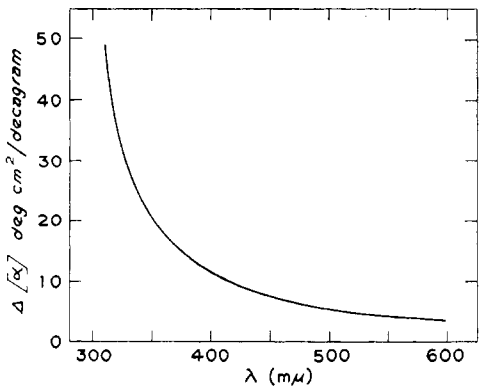

FIGURE 5: Difference optical rotatory dispersion for lysozyme in the presence and absence of *N*-acetyl-D-glucosamine. Concentrations: lysozyme, 0.857 g/dl in 0.10 M NaCl, and NAG, 0.156 M in 0.10 M NaCl. Molar ratio of NAG to lysozyme is 262:1.

Figure 2) calculated from eq 7, in which the artifact terms are negligible. Substitution of the data of curves 1 and 2 into eq 5 yields the calculated rotation for sucrose (curve 6, Figure 2; in arbitrary units). In this case $\Delta\alpha$ is simply the rotation α of sucrose solution.

The case in which solutions absorb was studied by placing potassium dichromate, which is optically inactive and nonfluorescing, in series (cells 2 and 4) with both the water and sucrose solution of the previous experiment. The resultant measured dispersion curves, 3 and 4 (Figure 2), did not coincide with those in the absence of absorption (curves 1 and 2), nor were they mirror images. Instead, both curves were more levorotatory than curves 1 and 2 with the maximum deviation near 350 mμ. Substitution of these data into eq 5 yields the calculated optical rotatory dispersion for sucrose (curve 5, Figure 2; in arbitrary units) without noticeable evidence of artifact rotation. At 350 mμ curve 5 differs from curve 6 by less than 3.5%.

The artifact rotations of curves 3 and 4 were completely associated with absorbing solutions in the r beam whether or not optically active. No artifact rotations were produced by potassium dichromate placed in the i beam whether or not there was appreciable absorption present in the r beam (see also Samejima and Yang, 1964). This is further illustrated in Figure 3, where a potassium dichromate solution placed in the r beam produces apparent levorotation of magnitude directly related to the optical density of the solution. Indeed, the profile of the artifact with respect to wavelength parallels the corresponding absorption spectrum. Figure 4 shows the false rotations produced by potassium dichromate solutions in the r beam at the absorption maximum of Figure 3 (~352 mμ). The magnitude of the false rotation is small below 0.5 optical density but increases sharply at higher optical densities.

During the course of this investigation our instrument (more than 3 years old) was serviced four times. Each servicing altered the magnitude of the false rotation but did not eliminate it. As an additional check, potassium dichromate solutions were measured with another Cary 60 which had been in use for less than 500 hr. The results were qualitatively the same as for our instrument. The vertical bars in Figure 4 represent the average variation of the false rotations observed for the two instruments on the five different occasions on which the magnitude of the artifact was specifically monitored. Thus the artifacts associated with the presence of absorption in the r beam as a practical matter appear to be a property of the instrument, though their quantitative aspects probably will vary with individual instrument alignment.

$\Delta[\alpha]$ *of Lysozyme–Inhibitor Complex.* X-Ray diffraction studies of lysozyme (Blake *et al.*, 1965; Johnson and Phillips, 1965) indicate that certain inhibitors and substrates bind to a cleft in the surface of the molecule. In particular lysozyme was shown to bind strongly one molecule of the inhibitor, NAG, specifically in the cleft. No other binding sites for NAG were detected. The noninhibitor, D-glucosamine, appeared not to bind specifically at one site but possibly at many. These interactions can be reflected in the difference rotation, since the binding of the inhibitor, NAG, induces a detectable change in the rotation of either NAG or lysozyme, or both. The difference optical rotatory dispersion of lysozyme, as calculated from eq 5, with its binding site(s) saturated with NAG is shown in Figure 5. The rotation in the visible region of the solution pair (LIS + S) was less levorotatory than the (LS + IS) pair. If the latter pair are arbitrarily taken as the "reference" solutions, the difference specific rotation $\Delta[\alpha]$ (calculated on the basis of the concentration of lysozyme alone) may be considered to be positive for these solutions, and indeed if they are placed in the r beam a net positive rotation is recorded. It should be recalled that the instrument gives a positive sign to the recorded output of a levorotatory solution placed in the r beam and *vice versa*.

In a separate experiment the data of Figure 5 were extended to 260 mμ with 1-mm cells to reduce absorp-

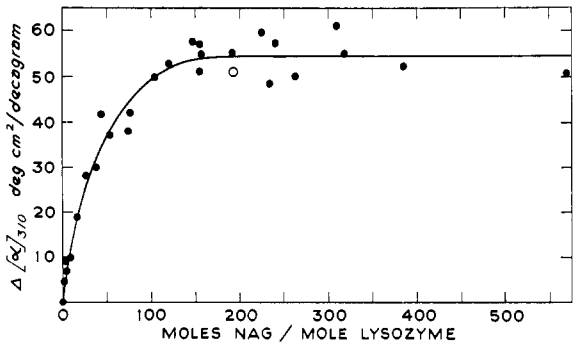

FIGURE 6: Difference specific rotation at 310 mμ of lysozyme–*N*-acetyl-D-glucosamine solutions as a function of molar mixing ratio. Concentration of lysozyme: 0.84 ± 0.03 g/dl in 0.10 M NaCl, except one point which was 0.421 g/dl (open circle).

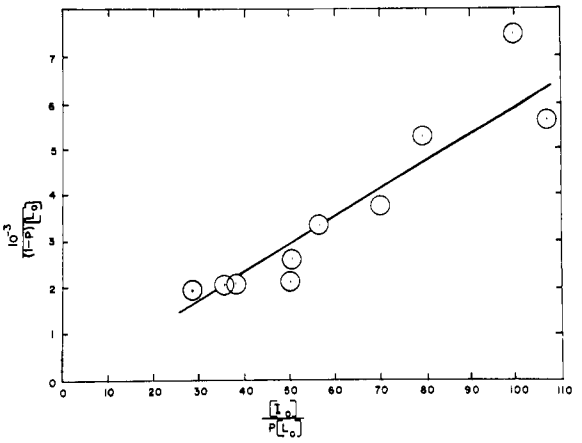

FIGURE 7: Plot of the binding data of lysozyme with *N*-acetyl-D-glucosamine. Based on data of Figure 6.

tion. Though not shown in Figure 5, a maximum in $\Delta[\alpha]$ was found at 297 ± 1 mμ. From this maximum down to 260 mμ the difference optical rotatory dispersion spectrum was complex, rather flat, and of small magnitude comparable to experimental error. The profile in Figure 5 thus suggests an increase in the positive Cotton effect in accord with the recent report of Glazer and Simmons (1966), who found that the circular dichroism spectrum in the aromatic absorption region was enhanced when lysozyme is complexed with NAG.

Summarized in Figure 6 is the difference specific rotation of lysozyme–NAG solutions as a function of the molar ratio of NAG to lysozyme. The lysozyme concentrations were approximately constant and fell within the range of 0.84 ± 0.03 g/dl. The variation of NAG concentration caused large variations in optical density, but the maximum at 310 mμ of the solutions and cells (each 1 cm) in either i or r beam did not exceed 0.54. The total maximum difference in optical density observed between the two beams was roughly ±0.01 unit. This small absorption difference observed between solutions of lysozyme and the lysozyme–NAG complex at 310 mμ is consistent with the absorption difference spectra reported by Hayashi *et al.* (1963). At 310 mμ the total optical density in both beams ranged from 0.43 to 1.1. Under the experimental conditions used, saturation of the binding site(s) with NAG as indicated by the plateau region of Figure 6 was complete at a molar ratio of about 150. The average of $\Delta[\alpha]$ values at higher ratios was approximately 54.4 deg cm²/decagram. The average deviation from this mean was approximately ±5.3%; the maximum deviation was ±6.6 deg cm²/decagram or ±12%. These data were obtained with several solutions prepared over several weeks and probably represent the maximum error to be expected with the method. Thus, while this should not of itself affect the accuracy of these measurements, their precision should have been considerably improved by use of the same stock solutions for an entire experiment. In one experiment (the open circle in Figure 6) the concentrations of all solutions were halved by dilution, thus maintaining the same molar ratio (194); the resultant decrease in $\Delta[\alpha]$ of 3.9 is still within experimental error, though larger effects might be expected below the plateau region.

In order to determine the effect of the noninhibitor, D-glucosamine, on the optical rotation of lysozyme a set of four determinations at molar mixing ratios of 24, 61, 153, and 382 moles of glucosamine per mole of lysozyme yielded, respectively, measured values for $\Delta[\alpha]$ of 1.3, 5.9, 8.3, and 6.9. Although these values are approximately the same as or less than the maximum deviations of Figure 6, they may be reflecting nonspecific binding of D-glucosamine to lysozyme (Johnson and Phillips, 1965).

From the data of Figure 6 an equilibrium constant k has been estimated for the association of NAG inhibitor (I) with n independent and equivalent sites on lysozyme (L). Adopting the nomenclature of Edsall and Wyman (1958), the probability P that any randomly chosen binding site on lysozyme is occupied is given by

$$\frac{\bar{\nu}}{n} = \frac{k[\mathrm{I}]}{1 + k[\mathrm{I}]} \tag{8a}$$

where $\bar{\nu}$ is the average number of occupied binding sites per molecule of lysozyme, and [I] is the molar concentration of unbound inhibitor. Assuming that the plateau region of Figure 6 represents full occupancy of lysozyme binding sites, the fraction of sites occupied is given by $\Delta[\alpha]/54.4$, which is also the probability that any randomly chosen binding site is occupied. Thus $P = \bar{\nu}/n = \Delta[\alpha]/54.4$. The free molar NAG concentration [I] was estimated by difference from the total molar NAG concentration $[\mathrm{I}_0]$ and the total molar lysozyme concentration $[\mathrm{L}_0]$: $[\mathrm{I}] = [\mathrm{I}_0] - \bar{\nu}[\mathrm{L}_0]$. Substitution for [I] and $\bar{\nu}$ in eq 8a and rearranging produces

$$\frac{1}{(1 - P)[\mathrm{L}_0]} = k\frac{[\mathrm{I}_0]}{P[\mathrm{L}_0]} - nk \tag{8b}$$

Presented in Figure 7 is a plot of $1/(1 - P)[L_0]$ *vs.* $[I_0]/P[L_0]$ for data where $[I_0]/[L_0] \leq 77$. The slope provides k directly, and the x intercept provides n directly. The line through the data points is consistent with $k = 6 \pm 1 \times 10^1$ M^{-1} and $n = 1$. However, n cannot be determined from this plot because of the sensitivity of the intercept to scatter in the data; the slope is relatively insensitive. The value determined for k is in only fair agreement with the corresponding dissociation constant of 4–6 $\times 10^{-2}$ M at pH 5.0 and an ionic strength of 0.1 reported by Dahlquist *et al.* (1966) from absorption difference spectra.

Discussion

Fortunately, the electrooptical cause of the artifacts described need not be known to obtain meaningful difference optical rotation. The sensitivity of the difference capability of the Cary instrument makes it a powerful tool for measuring small changes in optical rotation which heretofore were of marginal signal-to-noise ratio or impossible to measure. Indeed, in the lysozyme–NAG binding experiments (Figure 6) the average maximum observed change in rotation was only about 13% of the total direct optical rotation for lysozyme at 310 mμ. Thus it would have been extremely difficult and perhaps impossible to obtain similar data from the usual procedure for measuring optical rotation, which would have involved at least three direct measurements (solution pairs of LS + IS and LIS + S measured consecutively in the same pair of cell plus one solvent base line) and two subtractions of the three data points. By comparison the difference rotation method requires only two measurements and one subtraction and has the advantages of being more precise, sensitive, rapid, and convenient.

An estimation of the limits of resolution possible with the difference rotation technique can be obtained from the data of Figure 6. Using as a criterion the estimated maximum deviation of ± 6.6 deg cm^2/decagram for the data points in the plateau region, difference rotations of $\pm 0.0054°$ on the recording chart should readily be detected. If instead the average deviation, ± 2.9 deg cm^2/decagram, of the plateau region data points is used as a criterion, difference rotations of $\pm 0.0024°$ are detectable. Preparing all solutions by dilution from single stock solutions would by improving precision make even smaller rotations measurable. It should be possible to approach the apparent limiting sensitivity of the instrument at high optical density of about $\pm 0.002°$ or less.

Finally, the base-line term b is present in both eq 3 and 4. Consequently, simultaneously placing a solution in the i beam and the corresponding solvent in the r beam does not eliminate the base-line rotation of the instrument. A separate determination of the base line is necessary unless it is canceled by substitution of two measured values which include it into an expression such as eq 5.

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