Study of the Cotton Effect of Proteins with Double Monochromator Optics*

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Received January 22, 1962

To reduce stray light, the optical rotatory dispersion of proteins was studied with a spectropolarimeter having two monochromators in series. Serum albumin, aldolase, bacterial amylase, β-amylase, alcohol dehydrogenases from yeast and liver, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, enolase, myokinase, phosphoglucomutase, phosphoglucose isomerase, insulin, and the pituitary growth hormone all exhibited definite Cotton effects. The rotatory dispersion curves of these proteins had negative troughs at 230–235 mμ and positive peaks at about 220–225 mμ. The specific rotation at the minimum of the trough was between −7000 and −17,000°, and this value was approximately proportional to the constant $-b_0$ of the Moffitt-Yang equation.

The chief purpose of this study was to establish the reality of the anomalous rotatory dispersion phenomena which were observed with proteins in the far ultraviolet (UV) spectral range (Simmons and Blout, 1960; Simmons *et al.*, 1961; Jirgensons, 1962a). As pointed out recently by Urnes and Doty (1961), spurious Cotton effects may appear if the amount of substance in the polarized beam is too high. Such optical artifacts were observed also by the author of this paper while working with the ordinary one-monochromator spectropolarimeter with the wave lengths of 200–300 mμ (Jirgensons, 1962a,b). For example, solutions of serum γ-globulin exhibited several maxima and minima when 0.1–1.0% solutions were examined in 1.0-cm cells, and the discontinuities in the curves disappeared when 0.01–0.05% solutions were used. According to Urnes and Doty (1961), the artifacts are caused by stray light, *i.e.*, solutions of high absorbance eliminate light for which the monochromator is set while permitting stray light outside the band to reach the analyzer. For the same reason, the troughs in the rotatory dispersion curves at 230–240 mμ are shallower as more protein is placed in the beam (Jirgensons, 1962a). Either a double-prism monochromator or two single-prism monochromators in series might be expected to reduce stray light (Sawyer, 1944). This latter alternative was chosen in this work because two single-prism monochromators were already at hand. As shown in the Experimental section, the method still needs further refinement. However, already at the present stage of development it shows great promise as a micromethod for conformational analysis, since the rotatory power at the minimum of the Cotton effect is so great that 0.1–1 mg of protein is sufficient for the characterization.

* This study was supported in part by grants from National Cancer Institute, National Institutes of Health, U. S. Public Health Service (grant C-1785), The Robert A. Welch Foundation, Houston (grant G-051), and American Cancer Society (grants P-262 and P-263). The results of this paper were presented at the American Chemical Society National Meeting, Washington, D. C., March 23, 1962.

EXPERIMENTAL

The Proteins.—A four-times-recrystallized bovine *serum albumin* and a chromatographically purified human serum albumin (Jirgensons, 1962a) were used. According to paper electrophoresis tests, the albumins did not contain detectable amounts of globulin impurities. The albumins were dissolved either in water or in 0.01 M sodium chloride solutions. No differences could be found between the rotatory dispersion of the albumins with respect to species, ionic strength, or slight variation of *p*H. *Aldolase, alcohol dehydrogenase* from *yeast, alcohol dehydrogenase* from *liver, glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, enolase, myokinase, phosphoglucomutase,* and *phosphoglucose isomerase* all were crystallized analytical grade enzymes; they were products of the C. F. Boehringer und Soehne, GmbH, Mannheim, Germany, and they were obtained through the California Corporation for Biochemical Research, Los Angeles. The enzymes were obtained in the form of crystalline suspensions in salt solutions (chiefly ammonium sulfate), and they were dissolved by diluting the suspension with water. According to the specifications, the enzymes were free of significant amounts of contaminating proteins; they were transported by air mail, kept under refrigeration, and used at least 2 months before the specified expiration date. The sweet potato *β-amylase* was a three-times-crystallized product from Mann Research Laboratories, New York, and recrystallized *insulin* was obtained from the same company. The *bacterial amylase* was a gift from Dr. Kin-ichi Sugae; the enzyme was isolated from *Bacillus subtilis* cultures, and it was freshly recrystallized by Dr. Sugae. The crystals of the amylase were dissolved in a very diluted solution of calcium hydroxide (final *p*H 9.2), and the crystalline suspension of the β-amylase was dissolved by dilution

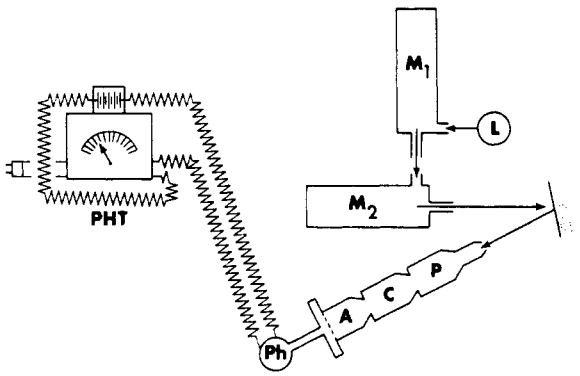

FIG. 1.—Schematic sketch of the spectropolarimeter. *L*, light source; M_1, first monochromator; M_2, second monochromator; *P*, rocking polarizer; *C*, cell compartment; *Ph*, photocell; *PHT*, photometer with batteries on top.

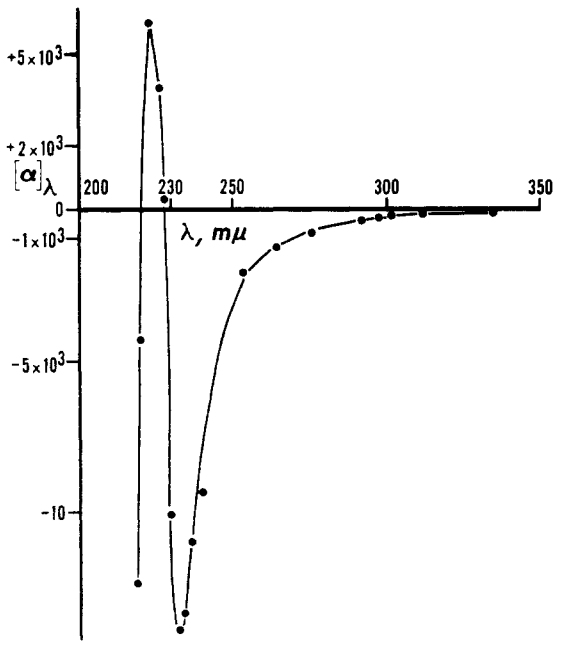

FIG. 2.—The Cotton effect of phosphoglucomutase. The specific rotation has a minimum of −14,700° at 232 mμ.

with water. Porcine *pituitary growth hormone* was a gift from Dr. A. E. Wilhelmi (Emory University, Atlanta, Georgia); the hormone was dissolved in 0.01 M sodium phosphate of *p*H 8.8, and insulin was dissolved in approximately 0.01 M mixture of sodium chloride and hydrochloric acid of *p*H 3.2. The ammonium sulfate concentrations of the above-mentioned enzyme solutions, after final dilution, were about 0.04–0.2 N. The concentration of the proteins was 0.01–0.2% in most cases, determined either spectrophotometrically or by micro-Kjeldahl nitrogen analyses of the precipitated more concentrated stock solutions. The concentration of albumin, moreover, was checked by gravimetric analysis. In those cases where the specific rotation of the protein at a certain wave length was known, the concentration of the stock solutions was determined by determining the rotatory power. For the study of the Cotton effect, usually the same 0.01 or 0.02% protein solution was measured with 0.2, 0.5, and 1.0 cm cells, and the per cent concentration of the protein was referred to the 1.0 cm polarized beam path in the solution.

The Rotatory Dispersion Measurements.—A schematic sketch of the instrument is presented in Figure 1. The basic unit is that of the O. C. Rudolph & Sons model 80-AQ6 instrument; however, the beam from the light source (*L*) is directed through two monochromators. The light first enters the Beckman monochromator (M_1) and from there enters the Rudolph model 655 monochromator (M_2), from which it is reflected into the rocking polarizer (*P*). The sensing unit contains an RCA 7200 photocell which was boosted with additional 90-450 volts from batteries. Several Hanovia high-pressure xenon lamps and mercury-xenon lamps were used as light sources with the aid of a 1000-watt voltage stabilizer and power supply, with provisions for magnetic stabilization of the arc. The slit width of both monochromators was set to 1.6 mm when the plain xenon lamp was used at 220–240 mμ; it was reduced to 1.0 mm when the more powerful (225-watt) mercury-xenon source was employed. Several series of measurements were made also with an Osram XBO 450-watt xenon lamp at slit widths of 1.0–1.6 mm. The oscillating polarizer was set to a symmetrical angle of 5° (and in a few series of measurements on albumin solutions a 10° angle was also used). Teflon cells of solution layer thickness of 0.1, 0.2, 0.5, and 1.0 cm were used, and the fused silica windows were tightened by means of plastic spacers. (These cells are provided by Rudolph Instruments Engineering Company, Little Falls, N. J.) The zero point of the empty instrument and of the cells filled with solvents was checked at various conditions. Readings were taken at 2–5 mμ increments of wave length, and the specific rotation was computed from an average of 5–15 readings at the same wave length after correction for the zero point. The reproducibility was found satisfactory within the wave length range of 235–250 mμ, but it was poor in the still shorter wave length region (see Results). Thus there is more confidence in the data about the negative trough than about the positive peak. Also it appeared that the prisms of the monochromators differed somewhat in their dispersing property in the far UV at the wave lengths below 230 mμ. All measurements were made at room temperature of 23–25°. It is possible that a two-prism monochromator with a minimum of optical elements would be more efficient than the present arrangement. Because of the electrical noise and small angles to be measured, which usually amount to only 0.03–0.1°, the error was about ± 10 to ± 15%

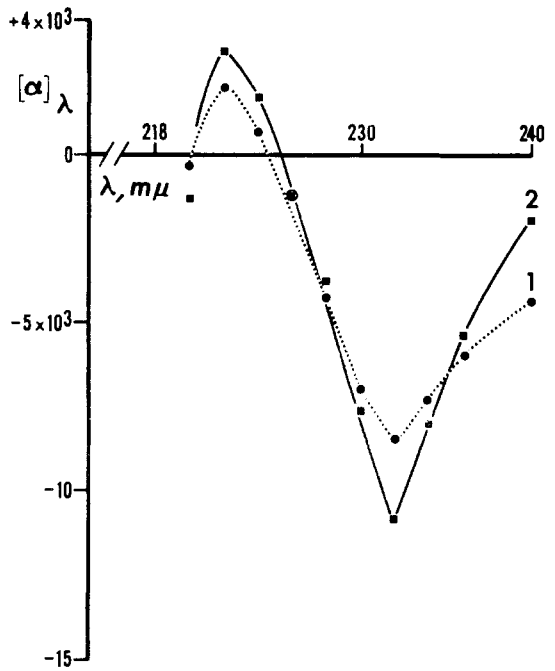

FIG. 3.—The Cotton effect of enolase. Curve 1, 0.01% enolase per 1-cm path; curve 2, 0.005% solution per 1-cm path. The open circle at 226 mμ is equivalent to the filled circles of curve 1.

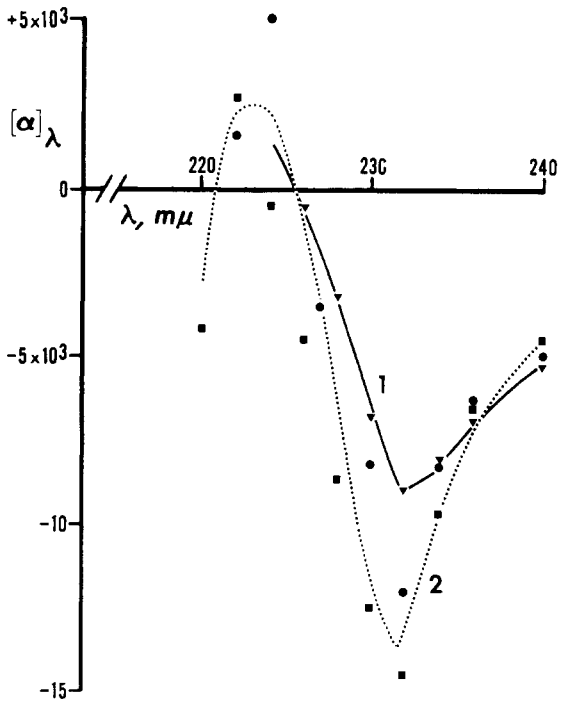

FIG. 4.—The Cotton effect of aldolase. Curve 1 (triangles), 0.01% aldolase per 1-cm path; curve 2, 0.004% enzyme per 1 cm. The squares and disks about curve 2 show the reproducibility at each wave length.

at the minimum of the Cotton effect and even larger in the 200–225 mμ range. Errors are involved also in the determination of the protein concentration, especially in the cases of enzymes which were available only in 2–5 mg quantities. Another source of error is the possible imperfection in the manufacture of the small cells of 1.00 and 2.00 mm. It is obvious that a recording attachment would be helpful, since many curves could be obtained in a short time and thus the reproducibility and errors could be evaluated more precisely than was possible with the present instrument.

RESULTS

The results are presented in Figures 2–7. Figure 2 illustrates a typical example of the rotatory dispersion in a relatively wide spectral range. The specific rotation is plotted against the wave length of light. The curve shows the rotatory dispersion of phosphoglucomutase, a muscle enzyme which catalyzes the isomerization of glucose-1-phosphate to glucose-6-phosphate. The measurements in the near UV were made with a 0.2% solution in a 1.0-cm cell, and at 220–250 mμ the optical activity was determined with a 0.02% solution in a 0.2-cm cell. The Cotton effect with a positive peak and a negative trough, with a minimum at 232–233 mμ, is characteristic, and the specific rotation at the minimum in this instance is −14,700°.

In Figure 3 is shown the rotatory dispersion of a 0.01% solution of enolase in the far UV. Curve 1 shows the rotatory dispersion of 0.01% enolase in the 1.0-cm cell and curve 2 the same solution in a 0.5-cm cell. Since there is a small difference in the Cotton effect, it seems that even the double-monochromator optics could not eliminate all of the stray light. This was confirmed with other examples, *e.g.*, Figure 4 illustrates the behavior of aldolase in concentrations 0.01% (curve 1) and 0.004% (curve 2) per 1.0-cm path. Moreover, the scatter of the points about curve 2 shows that the reproducibility, especially at the short wave lengths, is poor. The same can be said about the Cotton effect of serum albumin, which was studied extensively.

Since it was expected that the double monochromator system would eliminate the stray light, this dependence of the specific rotation on the amount of protein in the beam was questioned, and the rotatory dispersion of bovine and human serum albumins was repeatedly measured under the same or different conditions. Comparison of 16 series of measurement showed that the best reproducibility could be achieved by working with the mercury-xenon 225-watt lamp at 5° symmetrical angle and a slit width of 1.0 mm of both monochromators. The concentration dependence was ascertained, and the results are presented in Figure 5, where the specific rotation at the minimum of the trough is plotted versus logarithm of concentration (per cent concentration times 10^3,

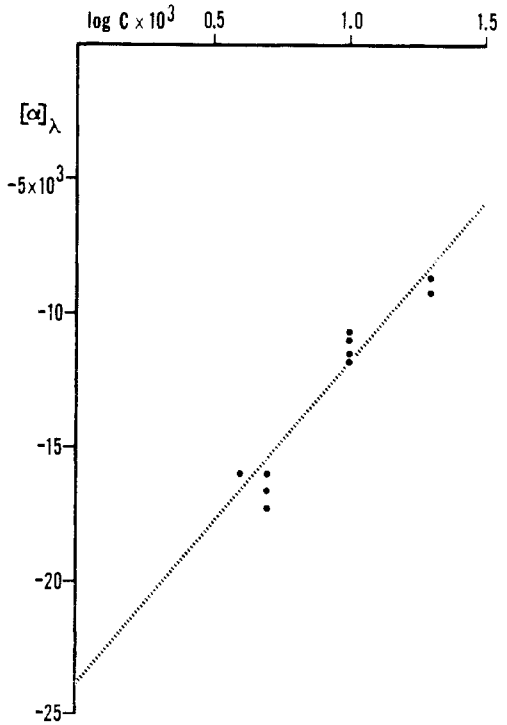

FIG. 5.—*Serum albumin.* Dependence of the specific rotation at the minimum of trough on the amount of protein in the beam. On the abscissa is plotted the logarithm of per cent concentration (times 10^3), and on the ordinate the specific rotation at a minimum of 232-233 mμ.

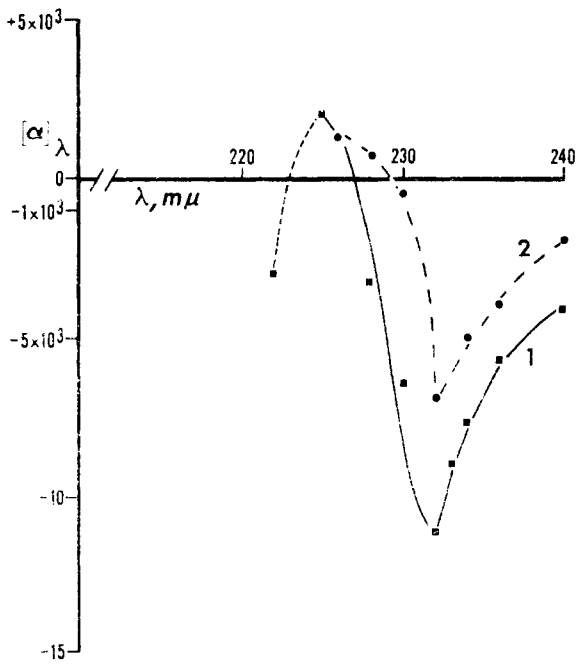

FIG. 6.—The Cotton effect of yeast alcohol dehydrogenase (curve 1) and liver alcohol dehydrogenase (curve 2). Amount of protein 0.004% per 1 cm in both cases.

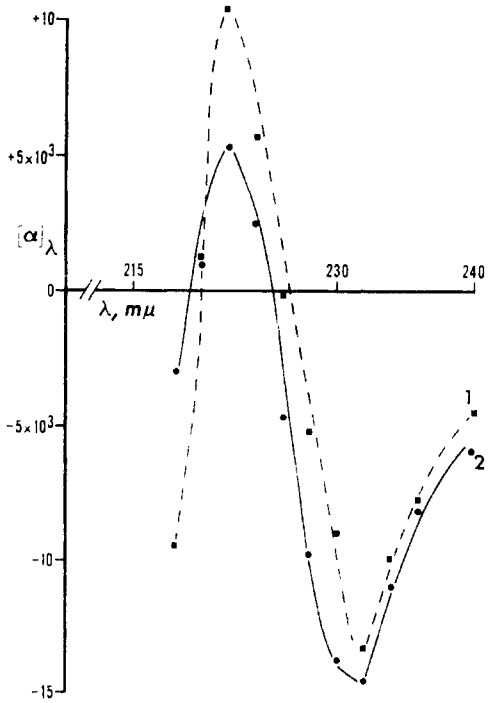

FIG. 7.—The Cotton effect of β-amylase (curve 1) and lactic dehydrogenase (curve 2). The points represent average values from three series of measurements in each case. Amount of protein in the beam 0.004–0.005% per 1-cm path.

as referred to 1.0-cm layer of solution). Because of the large scatter of the points, the straight line relationship is subject to doubt; but if extrapolation to zero amount is made, as suggested previously (Jirgensons, 1962a), the extrapolated specific rotation of albumin at the minimum is about −23,500°, whereas the single-monochromator technique yielded −18,500°.

In Figure 6 is shown the rotatory dispersion of the alcohol dehydrogenases from yeast and liver, respectively, and it is obvious that the enzymes differ significantly with respect to their Cotton effects. Finally, Figure 7 demonstrates the Cotton effects of β-amylase and lactic dehydrogenase. The curves represent in each case average values from three series of measurements on the enzyme solutions having 0.004–0.005% enzyme per 1.0-cm path. Measurements with more protein in the beam also were made, and a concentration dependence was observed with all proteins.

Definite Cotton effects were exhibited also by porcine pituitary growth hormone, insulin, bacterial amylase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglucose isomerase, and in all instances the troughs with the 0.01–0.02% protein solutions were shallower than with 0.004–0.005% protein per 1.0-cm path.

The results are summarized in Table I. In the first column are shown the directly observed values of the specific rotation at the minimum of the trough at 232-234 mμ. In the second column

TABLE I

SPECIFIC ROTATION OF SOME PROTEINS AT THE MINIMUM OF THE COTTON EFFECT (232–234 mμ) DETERMINED WITH THE DOUBLE-MONOCHROMATOR TECHNIQUE

Slit width 1.0 mm; symmetrical angle 5°; mercury-xenon 225-watt light source; concentration of protein 0.004–0.005% per cm solution layer (first column). The data are compared with the b_0 values (second column) and extrapolated values (see text).

	$-[\alpha]_{min}$ (2 monochrom.)	$-b_0$	$-[\alpha]_{min}^{c\to 0}$ (2 monochrom.)	$-[\alpha]_{min}^{c\to 0}$ (1 monochrom.)
Albumin, serum	17,200°	385	23,500° 19,300[	18,500°
Phosphoglucomutase	14,700	364		
Myokinase	17,300	300	23,000	12,000[a]
Growth hormone	13,400	276	19,200	18,500
Insulin	15,000	265	22,300 18,000[a]	
Lactic dehydrogenase	13,700	245	20,500 21,500[a]	13,300
Aldolase	12,600	229	18,100 14,000[a]	9,000
Phosphoglucose isomerase	12,700	209		
β-Amylase	13,300	195	17,000	13,000
α-Amylase, bacterial	10,400	192	18,400	10,000
Glyceraldehyde-3-phosphate dehydrogenase	10,700	172	15,000	9,900
Enolase	10,800	170	16,000	6,500
Alcohol dehydrogenase, yeast	11,200	155		
Alcohol dehydrogenase, liver	7,000	108		

[a] The variation illustrates reproducibility at somewhat different conditions.

are given the $-b_0$ constants of the protein. They were determined from the rotatory dispersion data in the near UV by using 0.1–0.2% solutions in 1.0, 5.0, or 10 cm tubes (slit width 0.3–0.5 mm), and plotting the $[\alpha]_\lambda \times (\lambda^2 - \lambda_0^2)$ values against $\lambda_0^4/(\lambda^2 - \lambda_0^2)$, where $[\alpha]_\lambda$ is the specific rotation at wave length λ, and λ_0 the Moffitt-Yang constant (=212 mμ) (Moffitt and Yang, 1956). (The refractive index and residual molecular weight factors were disregarded, since they do not affect b_0 greatly; if this correction is introduced the b_0 values of Table I should be reduced to about 10%.) In the third column of Table I are presented the specific rotation values at 232–233 mμ obtained on extrapolation of the specific rotation to zero concentration, and in the fourth column are given the same values as obtained earlier with the single monochromator optics (Jirgensons, 1962a).

DISCUSSION

A glance at the experimental data gives the impression that even the double-monochromator technique does not provide straightforward answers regarding the precise description of the Cotton effect. The reproducibility is not satisfactory, and not all stray light is eliminated. Thus the data are of only tentative value, and further improvements in the technique are called for. This is especially true for the shorter wave range of 200–230 mμ. The specific rotation values at the minimum of the Cotton effect, however, can be used now for consideration of conformation. The specific rotation values at this point determined with the present instrumentation are larger (more negative) than those obtained previously (Jirgensons, 1962a). This result would be anticipated from the modification in optics and light sources employed and from the use of lower protein concentrations. Thus, the present data appear more reliable than those previously reported (Jirgensons 1962a). The degree of precision of both the directly observed and the extrapolated values is low, so that the *intrinsic specific rotation* of the proteins is somewhat uncertain at the present time. Reduction of absorbancy of the sample by dilution further increases specific levorotation, and the extrapolated values may be closer to the true values than the directly measured quantities.

Comparison of the rotatory power in either column one or three of Table I with the b_0 constants indicates that the more negative the specific rotation at the minimum of the trough, the more negative generally is b_0. And since there is good evidence that b_0 can be correlated to the α-helix content (Moffitt and Yang, 1956; Urnes and Doty, 1961), it seems that the Cotton effect values are related to the α-helix content in the macromolecules, an assumption made first by Simmons and Blout (1960), and Simmons *et al.* (1961). These authors brought additional evidence in favor of this assumption by showing that the α-helical polyamino acids, *e.g.* poly-α-L-glutamic acid at *p*H 4.5, yielded a Cotton effect similar to that of some proteins, whereas the random chain polyamino acids gave smooth

curves. It was, however, surprising that the specific rotation values found by these authors for the helical polyamino acids or muscle proteins at 233 mμ were only about −12,000°. Since this value was obtained with the common single-monochromator optics, it was of considerable interest to make comparative measurements with the double-monochromator system. According to a personal communication from Dr. E. R. Blout, some of the helical polyamino acids were checked recently with a double-monochromator spectropolarimeter, and the substances had $[\alpha]_{233}$ values of −12,000 to −16,000°. This is only slightly higher (more negative) than the value obtained with the ordinary optics (Simmons *et al.*, 1961), and in the same range as found here with the globular proteins. Since the latter, according to their λ_c or b_0 values, can be only 30–70% α-helical, this posed a serious problem concerning the validity of the reasoning as well as possible imperfections in the experimental methods. Dr. Blout kindly sent a specimen of the α-helical poly-α-L-glutamate (sodium salt), and the rotatory dispersion of this polymer was measured with our spectropolarimeter having two monochromators in series. An aqueous stock solution of the polymer was prepared assuming 10% moisture in the sodium salt, and the *p*H of the solution was adjusted to 4.5 by adding hydrochloric acid. The stock solution then was diluted to 0.02% and the rotatory dispersion was measured in 1.0, 0.5, and 0.2 cm cells with monochromator settings of 230, 232, 233, 234, and 236 mμ. In agreement with the results of Dr. Blout's group, it was found that the specific rotation of the 0.02% solution in a 1.0-cm cell was −12,000°. However, higher values of −14,300° were found when there was 0.01% of the polymer per 1-cm path, and −15,500° for 0.004% of the substance per cm. These results enhance the confidence in the technique, but at the same time they expose new problems. If it could be assumed that the polyglutamic acid was not 100% α-helical, the reconciliation between the protein and polyamino acid data would be easier.

Since the present instrumentation, including the double monochromator, does not entirely solve the problem of accurate rotational measurements at shorter wave lengths, further improvements in the instrumentation will be beneficial. The difficulties could be overcome to some extent by extrapolation to zero concentration or zero path, but it is not yet well established how this extrapolation should be done. With proteins, the logarithmic plot yielded straight lines, although only 3–4 points usually were available and the fit was not excellent. In the instance of the poly-α-L-glutamic acid, however, a simple nonlogarithmic relationship between the $[\alpha]_{233}$ and concentration was found, and the slope of the line was small. This may be due to a lesser absorption of light by the polyamino acids than in proteins. Also it is possible that the dependence between the amplitude of the Cotton effect and conformation in the polyamino acids is somewhat simpler (or different) than in proteins. The amino acid side-chain effects, solvent effects, and the presence of other orders aside from the α-helix are some of the factors worthy of consideration.

In conclusion: the elimination of stray light is essential for the study of the Cotton effect of proteins. This elimination was partially achieved with the present instrument, but further improvements are essential. The rotatory dispersion curves here reported have positive peaks and deeper negative troughs than the curves obtained previously. The employment of a double monochromator does not yield better reproducibility than the ordinary apparatus; however, the presently constituted instrument with a double monochromator does appear to give more reliable rotational measurements at shorter wave lengths, presumably by reducing stray light. The directly observed specific rotation of proteins at the minimum of the trough at 232–233 mμ is between −7000 and −17,000°. These values are roughly proportional to the b_0 values, which are known to express the α-helix content. However, the directly found rotatory power is not the intrinsic specific rotation, because all of the stray light could not be eliminated. The values found by extrapolation to zero amount of protein are even more negative but the uncertainty of this extrapolation is so large that it is impossible to give accurate values at the present time.

The theoretical difficulties involved in understanding the rotatory dispersion as dependent on conformation have been discussed by several authors, notably Kauzmann (1957), Tanford *et al.* (1960), Schellman and Schellman (1961), and Urnes and Doty (1961). Moreover, some important points have been elucidated in recent papers of Leonard and Foster (1961) and Rosenheck and Doty (1961).

Acknowledgments

The author is indebted to Professor Paul Doty (Harvard University) and Dr. Elkan R. Blout (Polaroid Corporation, Cambridge, Mass.) for the preprints of their papers. Moreover, I am grateful to Dr. Blout for specimens of polyamino acids, and to Mr. Hellmuth Rudolph of Rudolph Instruments Engineering Company (Little Falls, N. J.) for his help in solving problems of instrumentation.

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Nuclear Magnetic Resonance Spectra of Porphyrins*

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Received April 17, 1962

The nuclear magnetic resonance spectra of twenty-two porphyrins in $CDCl_3$ solutions are reported. Included are metal complexes of Ni(II), Pd(II), and Zn(II) and a chlorin. Interpretations of effects of the spectra due to differences in the electron-withdrawing, magnetically anisotropic, and shielding characteristics of substituents, and in their relative positions, have led to detailed proton assignments of value in the characterization of natural porphyrins and their derivatives. Particularly important in these interpretations are long-range deshielding effects of magnetically anisotropic substituents and the effect of the ring-current field. The latter effect decreases with increasingly electron-withdrawing substituents (as reflected in decreasing nitrogen basicity) and upon metal complex formation, but is not greatly affected by chlorin formation.

Several reports on the n.m.r. spectra of porphyrins have recently appeared (Becker and Bradley, 1959; Ellis *et al.*, 1960; Becker *et al.*, 1961; Abraham *et al.*, 1961; Abraham, 1961). As in these spectra the different protons extend over a wide range of resonance frequencies and spin-spin interaction between groups at different positions on the ring is not observed, the spectra can be more readily interpreted than might be expected and thus constitute an extremely useful and much needed means for elucidating the structure of porphyrins and related compounds.

In this paper we wish to report our studies on the n.m.r. spectra of porphyrins. The porphyrins previously studied with one exception contained only substituents which have nearly equivalent "electron-withdrawing capacity" (simple alkyl groups, or hydrogen in the case of porphin). In this study compounds with strongly electron-withdrawing substituents are also included as are compounds of varying symmetry of substitution. Effects due to metal complex formation were noted and a chlorin (*i.e.*, dihydroporphyrin) has been compared with its corresponding porphyrin. These spectra were of interest both for characterizing new compounds, such as the green *a*-type heme associated with cytochrome oxidase (Caughey and York, 1962) and also as an extension of other studies of structural effects in porphyrins and metalloporphyrins (Caughey *et al.*, 1962; Alben and Caughey, 1962). Marked effects of changes in structure upon the strength of the ring current field and of long-range deshielding by magnetically anisotropic substituents have been observed, and a number of new assignments for protons commonly encountered among natural and synthetic porphyrins have been made.

EXPERIMENTAL

N.m.r. spectra were obtained with a Varian Associates spectrometer operating at 40 mc./second. Spectra were scanned from low to high field at rates of 70 to 300 cps/minute. The porphyrins were dissolved in $CDCl_3$ at a concentration $\sim$ 0.1 M. Tetramethylsilane (TMS) was used as an internal reference. The data are expressed in p.p.m. referred to tetramethylsilane as 10.00 (τ-values) according to Tiers (1958).

* This work was supported by grants from the U. S. Public Health Service (H-6079 and RG-5144).