Analysis of the Circular Dichroism of the Complexes of 8-Anilino-1-naphthalenesulfonate with Bovine Serum Albumin†

Ezra Daniel‡ and Jen Tsi Yang*

ABSTRACT: The induced circular dichroic (CD) spectra of anilinonaphthalenesulfonate bound to bovine serum albumin were resolved into four component rotational transitions. The change in the CD spectrum with the average number of bound ligands is shown to involve only two of the component bands and is attributed solely to coupling of transitions in the bound anilinonaphthalenesulfonate molecules. Under conditions of stoichiometric binding, the observed CD spectra could be represented as a superposition of two basic spectral components, one resulting from interaction of the ligand with the protein and the other from a pairwise interaction of bound ligands. The interpretation of the findings is consistent with the presence of essentially equivalent binding sites for anilinonaphthalenesulfonate on albumin, a conclusion obtained previously from direct fluorimetric titrations.

Fluorescent probes such as anilinonaphthalenesulfonate (ANS)[1] and related dyes have come into frequent use lately for the assessment of hydrophobicity of binding sites on proteins and as a means of monitoring conformational changes in biological macromolecules (Stryer, 1965, 1968). Recently, it has been suggested that the extrinsic Cotton effects induced in dyes upon binding to proteins may be useful in the exploration of local protein conformation and thereby complement the information obtained from fluorescence emission properties (Hsu and Woody, 1971). The usefulness of such probes rests however on an understanding of the factors that affect the induced circular dichroic activity. An important step in this direction was the description by Anderson (1969) of the induced circular dichroic (CD) spectra of ANS bound to BSA, a system extensively studied by Weber and his coworkers (Weber and Young, 1964; Daniel and Weber, 1966; Weber and Daniel, 1966; Anderson and Weber, 1969). Anderson was, however, led to the conclusion that the ANS binding sites on BSA were heterogeneous, a result which could hardly be reconciled with earlier findings from direct fluorimetric titrations carried out on this system (Daniel and Weber, 1966).

In this study a reexamination of the optical activity of ANS–BSA complexes was carried out. A consideration of both absorption and circular dichroic spectra led to a more detailed resolution of the electronic spectrum of ANS than has hitherto been possible. As a result a new interpretation of the experimental data, consistent with current ideas of the properties of the ANS–BSA system, is proposed.

Experimental Section

Crystallized BSA was purchased from Armour Pharmaceutical Co. and the magnesium salt of 8-anilino-1-naphthalenesulfonic acid was a product of Eastman Kodak. Buffer reagents were analytical grade. The preparation of solutions and the measurement of concentrations were as described elsewhere (Daniel and Weber, 1966).

Circular dichroism was measured with a Durrum-Jasco recording spectropolarimeter, Model J-10. Constant temperature was maintained by circulation of water in a specially designed cell holder from a Haake thermostated bath. Optical cells with fused silica windows and with path lengths ranging from 20 to 0.1 mm were used. The absorbance of the solutions was kept below two. Absorption spectra were determined in a Zeiss PMQ II spectrophotometer.

The absorption and CD of ANS in ANS–BSA complexes were determined by carrying out parallel measurements on albumin–dye mixtures and equivalent solutions containing albumin only. In this way, the direct contribution of the protein was subtracted. Circular dichroism and absorption were expressed in terms of molar circular dichroic absorptivity $\epsilon_l - \epsilon_r$ and molar extinction coefficients ϵ (based on the total concentration of ANS).

Results

Figures 1 and 2 show the absorption and CD spectra of ANS in ANS–BSA complexes. The absorption spectrum of the bound ANS is practically invariant of the number of ligand binding sites occupied, $\bar{n}$, under conditions of stoichiometric binding. However, some of the CD bands show variations with $\bar{n}$. Thus, while $\epsilon_l - \epsilon_r$ at $\lambda \geq 380$ nm is, within experimental errors, independent of $\bar{n}$, $\epsilon_l - \epsilon_r$ at $300 < \lambda < 380$ nm increases with $\bar{n}$. Anderson (1969), however, found that both the 340- and 380-nm bands changed with $\bar{n}$. Figure 3 shows that $(\epsilon_l - \epsilon_r)_{340}$ is a linear function of C_{ANS}/C_{BSA} up to a concentration ratio of 4, which is the range of stoichiometric binding under the experimental conditions. Another straight line follows at $C_{ANS}/C_{BSA} > 5$. A decrease in $\epsilon_l - \epsilon_r$ at about 272 nm, parallel to the increase at 340 nm, is also apparent (Figure 3, inset).

† From the Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94122. *Received August 20, 1972.* This work was aided by U. S. Public Health Service Grants HL-06285 and GM-10880.

‡ On leave from the Biochemistry Department, Tel-Aviv University, Israel.

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; BSA, bovine serum albumin; $\bar{n}$, average number of moles of ligand bound per mole of protein.

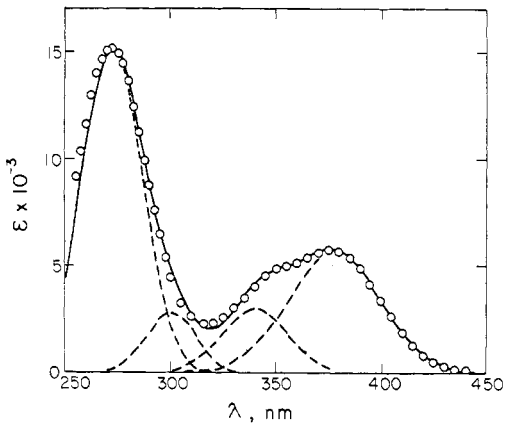

FIGURE 1: Absorption spectrum of ANS bound to BSA (C_{BSA} = 1.5×10^{-4} M; $C_{ANS} = 1.5 \times 10^{-4}$ M) in 0.1 M phosphate buffer (pH 7.0). (O) Observed values; (—) sum of the component bands (– – –).

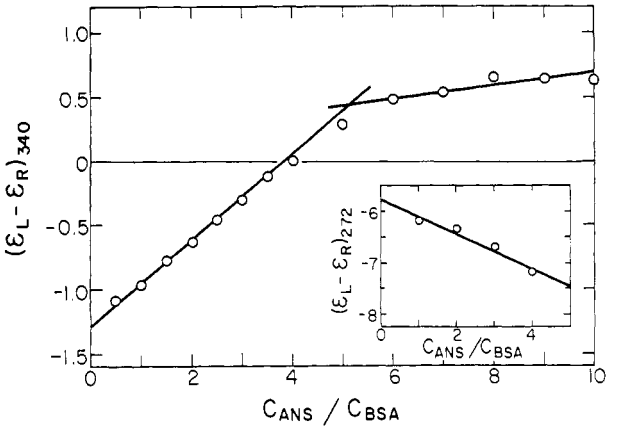

FIGURE 3: Plot of $(\epsilon_l - \epsilon_r)_{340}$ and $(\epsilon_l - \epsilon_r)_{372}$ (inset) *vs.* C_{ANS}/C_{BSA}. Conditions: 0.1 M phosphate, pH 7.0, 25°, and $C_{BSA} = 1.5 \times 10^{-4}$ M throughout. For $C_{ANS}/C_{BSA} \leqslant 4$, virtually all the ANS present in solution is bound to the protein and $\bar{n} = C_{ANS}/C_{BSA}$.

Interpretation of the results requires the identification of the separate electronic transitions which contribute to the induced optical activity of the bound ANS. Resolution of complex CD spectra such as those shown in Figure 2 is best carried out by simultaneous consideration of the corresponding absorption spectrum (Holzwarth and Doty, 1965; Miles and Urry, 1968a). It was found that a resolution into four gaussian bands provided a solution consistent with both absorption (Figure 1) and CD (Figure 2) spectra. The locations and magnitudes of the component bands are summarized in Table I.

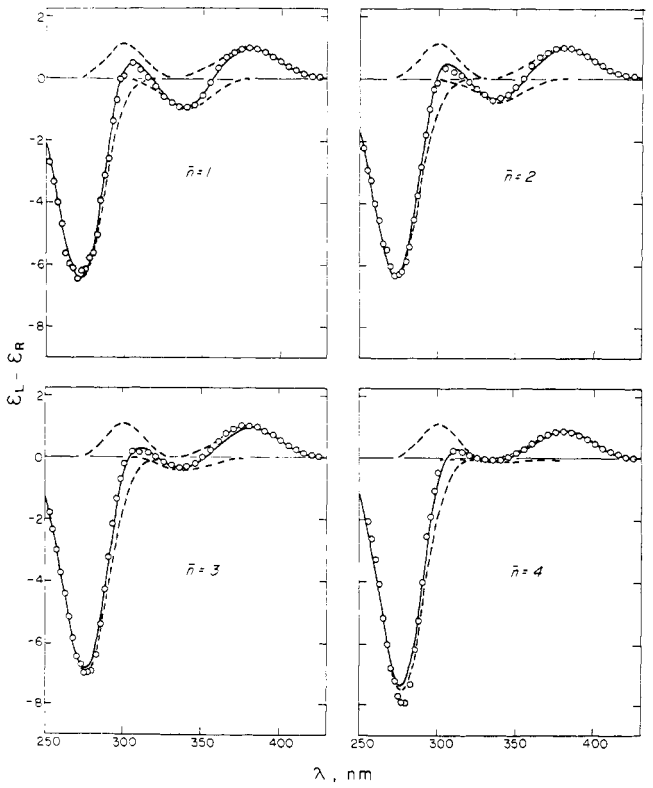

FIGURE 2: Circular dichroism spectra of ANS bound to BSA, $\bar{n} = 1$ to $\bar{n} = 4$, in 0.1 M phosphate buffer (pH 7.0) at 25°. $C_{BSA} = 1.5 \times 10^{-4}$ M throughout. (O) Observed values; (——) sum of the component rotatory bands (– – –).

At $C_{ANS}/C_{BSA} > 5$, appreciable concentrations of free ANS, relative to that of bound ANS, occur. A typical example is that of a solution in which $C_{ANS}/C_{BSA} = 10$. Since free ANS is optically inactive, the experimental ellipticity per unit light path, which is equivalent to $(\epsilon_l - \epsilon_r)C_{ANS}$, is not expected to be appreciably different from the one predicted for $\bar{n} = 5$, where all five binding sites are occupied. A comparison (Figure 4) reveals, however, appreciable differences in the corresponding curves, and additional factors that affect the CD spectrum must be sought (see Discussion).

Discussion

Resolution of the absorption and CD spectra of ANS in ANS–BSA (Table I) shows that three of the four component bands have identical positions and bandwidths in both absorption and CD (Moffitt and Moscowitz, 1959). The

TABLE 1: Optical Parameters of Gaussian Component Bands Obtained in the Resolution of the Absorption and CD Spectra of ANS in ANS–BSA Complexes.

Absorption Bands			Rotatory Bands[a]		
Wavelength of Extremum (nm)	$\epsilon \times 10^{-3}$ (cm²/mmol)	Oscillator Strength	Wavelength of Extremum (nm)	$\epsilon_l - \epsilon_r$ (cm²/mmol)	Rotational Strength[b] $\times 10^{40}$ erg cm³ rad
378	5.66	0.09	380	1.00	2.9
340	2.95	0.04	340	−1.33	−2.5
300	2.80	0.04	300	1.12	2.6
272	15.2	0.30	272[c]	−6.50	−19.4

[a] Values obtained at $\bar{n} = 1$. [b] The rotational strength, R_i, was calculated from the relation (Moscowitz, 1960): $R_i = 1.23 \times 10^{-42}[\theta_i^0]\Delta_i^0/\lambda_i^0$, where $[\theta_i^0] = (2.303)(4500/\pi)(\epsilon_l - \epsilon_r)_i^0$ is the molar ellipticity of the ith transition at the wavelength of the extremum, λ_i^0, and Δ_i^0 is the half-bandwidth at $(\epsilon_l - \epsilon_r)_i^0/e$. [c] The position of this component band is slightly red shifted for $\bar{n} > 1$.

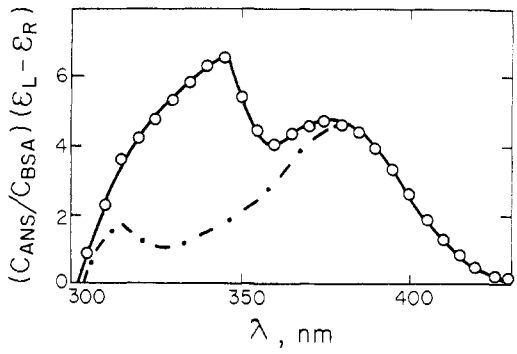

FIGURE 4: Comparison of (C_{ANS}/C_{BSA}) $(\epsilon_l - \epsilon_r)$ of ANS at $C_{ANS}/C_{BSA} = 10$ (○) and the expected values for $\bar{n} = 5$ (-···-) (see Figure 6). Conditions: 0.1 M phosphate, pH 7.0, 25°, and $C_{BSA} = 1.5 \times 10^{-4}$ M.

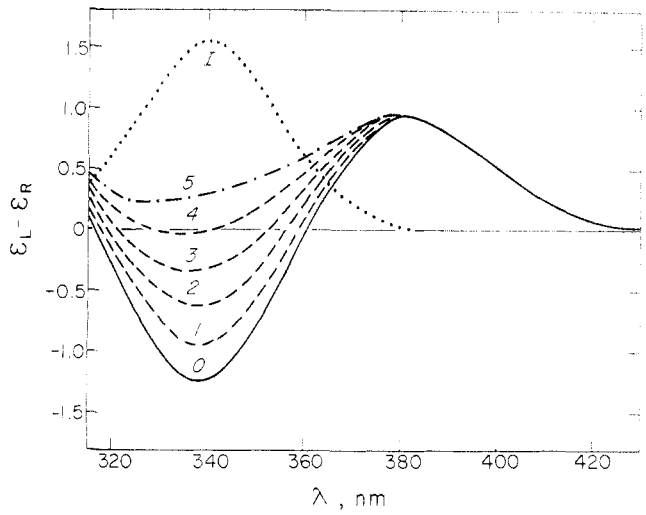

FIGURE 6: Generation of the CD spectra of bound ANS from two basic components, (—) $(\epsilon_l - \epsilon_r)_0$ and (···) $(\epsilon_l - \epsilon_r)_1$. The spectra for $\bar{n} = 1$ to $\bar{n} = 4$ (-·-·-) agree with the solid lines in Figure 2. The spectrum for $\bar{n} = 5$ (-···-) is the limiting spectrum expected for complete saturation.

longest wavelength absorption band is, however, broader and located at shorter wavelengths than the corresponding CD band. This suggests that the longest wavelength transition in ANS may have a partial contribution from the excitation of a lone-pair electron on the nitrogen ($1 \rightarrow a_\pi$ transitions, intermediate in character between $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, are possible in derivatives of aromatic amines (Kasha, 1961; Kasha and Rawls, 1968)). A study of the fluorescence polarization spectrum of ANS in ANS–BSA allowed Anderson and Weber (1969) to resolve the long-wave portion of the ANS spectrum into two component bands with maxima at 378 and 345 nm. A comparison with Table I shows that the resolution derived from consideration of the CD spectrum is in good agreement with the one obtained on the basis of the spectrum of fluorescence polarization.

The dependence of the CD spectrum of bound ANS on $\bar{n}$ was interpreted by Anderson (1969) as evidence for the heterogeneity of the ANS binding sites in BSA. The detailed delineation of the electronic spectrum of ANS in the present study leads to a different interpretation of the factors that cause the observed changes in the CD spectra. Figure 2 shows that as $\bar{n}$ increases, the band at 340 nm becomes more positive and the band around 272 becomes more negative.

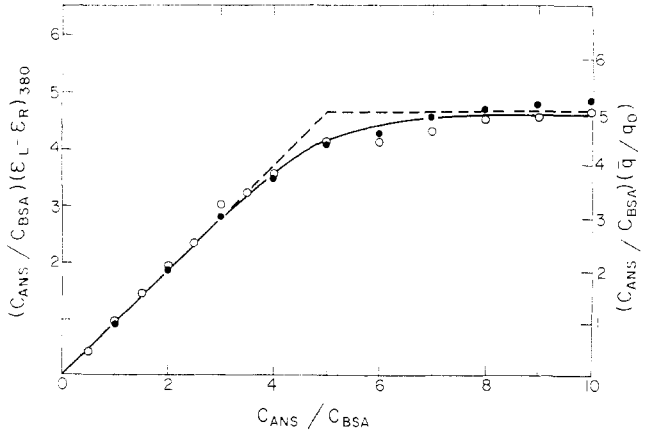

FIGURE 5: Stoichiometric plot of ANS binding to BSA. $C_{BSA} = 1.5 \times 10^{-4}$ M throughout. (○) Observed values of $(C_{ANS}/C_{BSA})(\epsilon_l - \epsilon_r)_{380}$; (●) relative fluorescence efficiencies (taken from Table I. Daniel and Weber, 1966); (—) theoretical binding curve for five binding sites with an apparent dissociation constant $K_D = 1.6 \times 10^{-6}$. The equivalence point is indicated by the intersection of the tangents (– – –).

This pairwise coupling of the 340- and 272-nm transitions strongly suggest that the spectral changes observed are a manifestation of reciprocal relations in optical rotation, a special case of the sum rule (Kuhn, 1930; Condon, 1937) theoretically discussed by Urry (1965) and Urry and Eyring (1966) and utilized by Miles and Urry (1967, 1968a,b) in the determination of conformational relationships in coenzymes and other small molecules. The way in which the rotatory power varies with $\bar{n}$ provides a clue to the nature of the interaction responsible for the optical effect observed. The linear dependence on C_{ANS} at constant C_{BSA} of $\epsilon_l - \epsilon_r$ for the 340-nm transition (Figure 3) is equivalent to a relation of the form (up to $C_{ANS}/C_{BSA} \sim 5$): $\psi = a_1 C_{ANS} + b_1 C_{ANS}^2$, where ψ is the observed ellipticity (per unit light path) and a_1 and b_1 are constants. If the binding is of the simple type—which is the case for ANS–BSA under our experimental conditions (Daniel and Weber, 1966)—this relation can be written as $\psi = a_2\phi + b_2\phi^2$, where a_2 and b_2 are constants and ϕ is the probability of binding to a given site. A dependence on the second power of ϕ means, however, that the dependent variable measures the probability that two sites be occupied at one and the same time. In other words, the reciprocal relations of the 340- and 272-nm bands result from the coupling of transitions on two ANS ligands bound to the same albumin molecule. One may postulate that out of the five ANS binding sites in BSA, two are singled out by the fact that their double occupancy allows the two bound ligands to interact, probably by bringing them in proximity to each other.[2] The present interpretation of the CD spectra is therefore fully consistent with the existence of essentially equivalent binding sites for ANS in BSA, a conclusion

[2] In principle, any of the ten pairs which can be formed out of the five albumin binding sites might be suitably arranged so as to bring the bound ligands into interactions with one another. However, the average distance between a pair of ANS binding sites in BSA is 21 Å (Weber and Daniel, 1966; Anderson and Weber, 1969), and the number of interacting pairs must consequently be smaller than the maximal one which is *a priori* possible. What our experiments unequivocally show is that at least one such interacting pair exists.

previously obtained from analysis of the fluorimetric titration data (Daniel and Weber, 1966).

The $\epsilon_l - \epsilon_r$ of the 380- and 300-nm component bands of bound ANS are, in contrast, independent of $\bar{n}$ and result from the interaction of bound ANS with the protein binding sites (first-order dependence of ψ on ϕ). Such component bands may therefore be used to study the binding process in the same way that other spectroscopic properties, such as absorbance or fluorescence emission, are used. Figure 5 shows a stoichiometric plot of the binding using the 380-nm centered band. The observed values are seen to be in satisfactory agreement with the description of the binding process obtained fluorimetrically.

Realization of the fact that two kinds of interaction affect the induced optical activity of bound ANS—the ligand–protein and the ligand–ligand interactions—suggests that a separation of their respective contributions to the measured CD spectra could be carried out. Figure 6 demonstrates that the observed CD spectrum at a given value of $\bar{n}$ can be obtained as a linear combination of two basic spectral components: $(\epsilon_l - \epsilon_r)_{\bar{n}} = (\epsilon_l - \epsilon_r)_0 + (\bar{n}/5)(\epsilon_l - \epsilon_r)_I$. Component $(\epsilon_l - \epsilon_r)_0$ is the limiting spectrum calculated for $\bar{n} \rightarrow 0$ and results from interaction of bound ligand with the protein (*cf.* $(\epsilon_l - \epsilon_r)_{340}$ extrapolated to $\bar{n} \rightarrow 0$ in Figure 3 with $(\epsilon_l - \epsilon_r)_0$ at $\lambda = 340$ in Figure 6). Component $(\epsilon_l - \epsilon_r)_I$ represents the contribution from ligand–ligand interaction.

Application of the method of matrix rank analysis (Wallace and Katz, 1964) to the ANS-induced CD spectra (Anderson, 1969) indicated the presence of two spectrally distinguishable components within the set of five primary binding sites. (A third component was attributed to binding at secondary sites with binding affinity much weaker than the first five.) Matrix rank analysis of our data for $\bar{n} \leq 4$ shows more definitely the presence of only two spectral components. The significance of this result is that the minimum number of independent sets required to satisfy the observed spectral data, compatible with expected experimental error, is two. This conclusion is consistent with the finding that the set of CD spectra can be generated from the two basic components $(\epsilon_l - \epsilon_r)_0$ and $(\epsilon_l - \epsilon_r)_I$.

Inspection of Figures 3, 4, and 6 leads to the conclusion that at $C_{ANS}/C_{BSA} > 5$ additional dye molecules do bind to the ANS–BSA complexes. One possibility is that albumin has additional binding sites—much weaker than the first five—for ANS (Anderson, 1969; see also Pasby and Weber, quoted by Weber, 1970). Additional binding sites on the albumin would however be hard—although by no means impossible—to reconcile with the finding that the change which the CD spectrum undergoes affects some bands only (the 272- and 340-nm bands), leaving at least one band (the 380-nm band) unaffected at all. We therefore propose that the CD changes that occur after the five binding sites had been saturated are caused by the association of additional ANS molecules with the ANS already saturating the protein binding sites. The invariability of the 380-nm band would then find a logical interpretation in the absence of further dye–protein interaction, while the changes affecting the 272- and 340-nm bands could still be attributed to additional dye–dye coupling. The explanation proposed here finds support in the observation that dye molecules that show aggregation in solution are usually ones that exhibit extrinsic Cotton effects (Stryer and Blout, 1961). Indeed there is evidence that at high concentration ANS associates to dimers and higher aggregates in solution (Muesing and Nishida, 1971).

Finally it may be pointed out that the ligand–ligand interaction responsible for the $\bar{n}$-dependent changes in the CD spectrum should not be confused with homotropic ligand–ligand interaction in the sense used by Monod *et al.* (1965) to describe deviations from statistical binding equilibria. In fact, the present study demonstrates that it is possible to have one without the other—ANS–ANS interaction that affects the optical properties in a system where direct titration studies have shown random occupancy of essentially equivalent sites as far as ligand binding is concerned.

Acknowledgment

We thank Mr. Yee-Hsiung Chen and Miss Barbara Wells for several helpful discussions.

Addendum

In a recent study which has come to our attention after this manuscript was submitted for publication, Brand and his coworkers (Seliskar *et al.*, 1969; Seliskar and Brand, 1971) presented evidence that the lowest energy transition in *N*-arylaminonaphthalenesulfonates is an $1 \rightarrow a_\pi$ transition. The findings of the present study provide therefore independent support, in the case of ANS, for the correctness of their assignment.

References

Anderson, S. R. (1969), *Biochemistry 8*, 4838.
Anderson, S. R., and Weber, G. (1969), *Biochemistry 8*, 371.
Condon, E. U. (1937), *Rev. Mod. Phys. 9*, 432.
Daniel, E., and Weber, G. (1966), *Biochemistry 5*, 1893.
Holzwarth, G., and Doty, P. (1965), *J. Amer. Chem. Soc. 87*, 218.
Hsu, M. C., and Woody, R. W. (1971), *Biopolymers 10*, 1421.
Kasha, M. (1961), *in* Light and Life, McElroy, W. D., and Glass, R., Ed., Baltimore, Md., Johns Hopkins University Press, p 31.
Kasha, M., and Rawls, H. R. (1968), *Photochem. Photobiol. 7*, 561.
Kuhn, W. (1930), *Trans. Faraday Soc. 26*, 293.
Miles, D. W., and Urry, D. W. (1967), *J. Phys. Chem. 71*, 4448.
Miles, D. W., and Urry, D. W. (1968a), *Biochemistry 7*, 2791.
Miles, D. W., and Urry, D. W. (1968b), *J. Biol. Chem. 243*, 4181.
Moffitt, W., and Moscowitz, A. (1959), *J. Chem. Phys. 30*, 648.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Moscowitz, A. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., Ed., McGraw-Hill, New York, N. Y., p 150.
Muesing, R. A., and Nishida, T. (1971), *Biochemistry 10*, 2952.
Seliskar, C. J., and Brand, L. (1971), *J. Amer. Chem. Soc. 93*, 5405.
Seliskar, C. J., Turner, D. C., Gohlke, J. R., Brand, L. (1969), *in* Molecular Luminescence, Lim, E. C., Ed., New York, N. Y., Benjamin, p 677.
Stryer, L. (1965), *J. Mol. Biol. 13*, 482.
Stryer, L. (1968), *Science 162*, 526.
Stryer, L., and Blout, E. R. (1961), *J. Amer. Chem. Soc. 83*, 1411.
Urry, D. W. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 640.
Urry, D. W., and Eyring, J. (1966), *Perspect. Biol. Med. 9*, 450.
Wallace, R. M., and Katz, S. M. (1964), *J. Phys. Chem. 68*, 3890.

Weber, G. (1970), *in* Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association, p 23.

Weber, G., and Daniel, E. (1966), *Biochemistry 5*, 1900.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239*, 1415.

Increase in the Stability of Avidin Produced by Binding of Biotin. A Differential Scanning Calorimetric Study of Denaturation by Heat†

John W. Donovan* and Kenneth D. Ross‡

ABSTRACT: Transition temperatures (T_{tr}) and enthalpies of denaturation (ΔH_{tr}) for avidin and the avidin–biotin complex were obtained by differential scanning calorimetry. Measurements near pH 7 and 9 indicated little effect of pH, ionic strength, or buffer ion on T_{tr} or ΔH_{tr}. Large increases in both T_{tr} and ΔH_{tr} resulted when avidin bound biotin. T_{tr} increased from 85 to 132°. The increase in ΔH_{tr}, from 300 to 1000 kcal per mol of avidin tetramer, appears to be due primarily to a large increase in heat capacity of the protein upon irreversible denaturation.

The stability of avidin and its complex with biotin has been the subject of several investigations (Green, 1966; Wei and Wright, 1964) since binding of biotin by avidin prevents assimilation of this vitamin (Eakin *et al.*, 1940, 1941; György *et al.*, 1941). The dissociation constant of the complex has been reported to be 10^{-15} M (Green, 1963a). Each of four biotin molecules is bound to one subunit of the avidin tetramer, and the binding appears to be noncooperative (Green, 1966). Wei and Wright (1964) have found that although avidin is heat labile at 100°, the complex is stable at that temperature—indeed, autoclave heating at 120° for 15 min was required to release bound biotin. Avidin and its complex with biotin are stable in 9 M urea, and over a pH range from 2 to 13.7 (Green, 1963b). A concentration of 7 M guanidine hydrochloride, just sufficient to denature avidin, does not denature the avidin–biotin complex (Green, 1963b). However, the denaturation temperatures of avidin and the avidin–biotin complex and the enthalpies of denaturation have never been determined.

We have employed the method of differential scanning calorimetry (DSC) to quantitatively evaluate the heat stability of avidin and the avidin–biotin complex. We carried out these studies at pH 9 and 7, since egg white and whole egg normally are at these pH values when they are heat processed in commerce (*e.g.*, during pasteurization or drying). Ample evidence exists to demonstrate the usefulness of DSC for the study of thermal denaturation of proteins (Beck *et al.*, 1965; Delben *et al.*, 1969; Tsong *et al.*, 1970; Jackson and Brandts, 1970; Crescenzi and Delben, 1971) as heats of transition can be quantitated more easily by DSC than by the older differential thermal analysis technique (Steim, 1965). Hermetically sealed containers which will withstand internal pressures of several atmospheres make possible determination of transition temperatures above the boiling point of water, such as those we have observed for the denaturation of the avidin–biotin complex.

Experimental Section[1]

Materials. Avidin was prepared by a variation of the method of Green and Toms (1970). Difference absorbance assay (Green, 1963a) yielded a value of 15.0 μg of biotin bound per mg of this preparation of avidin. The *d*-biotin was Calbiochem lot 000920 used without further purification. Ribonuclease was Worthington Biochemical Corp. lot R7LE. Lysozyme was Difco Labs. lot 0465-10. Tris(hydroxymethyl)-methylaminopropanesulfonic acid (Taps)[2] buffer was Sigma Chemical Co. lot 99B-5230. Pure indium was supplied by the Instrument Products Division of E. I. duPont de Nemours & Co. Benzoic acid was a National Bureau of Standards standard sample. The other substances used for calibration of the differential scanning calorimeter, the purest commercial material locally available, gave thermograms indicative of high purity.

Methods. Protein solutions used for quantitative measurement of denaturation are listed. Protein solutions at other concentrations were used in preliminary experiments to determine transition temperatures: for avidin, (a) 73.5 mg/ml at pH 6.84 and (b) 16.4 mg/ml at pH 6.62 in 0.1 M KCl–0.02 M in potassium phosphate; (c) 14.6 mg/ml at pH 9.04 in 0.1 M KCl–0.02 M borate; (d) 35.9 mg/ml at pH 9.14 in 0.1 M KCl plus universal buffer at half the concentration specified by Britton and Welford (1937). For avidin plus biotin, a stoichiometric excess of biotin (crystals) was added to an aliquot of each of a–d. The amount of biotin added exceeded its solubility at room temperature, so the solutions were drawn

† From the Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Berkeley, California 94710. *Received November 17, 1971.*

‡ Present address: Department of Chemistry, University of California, Berkeley, Calif. 94720.

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

[2] Abbreviation used is: Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid.