Optical Rotatory Dispersion, Circular Dichroism and Far-Ultraviolet Spectra of Avidin and Streptavidin

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1. The optical-rotatory-dispersion and circular-dichroism curves of avidin showed positive Cotton effects centred at $228\text{m}\mu$ and $280\text{m}\mu$, close to the ultra-violet-absorption bands of tryptophan. These effects disappeared when avidin was dissociated into sub-units in guanidine hydrochloride. 2. Binding of biotin had only a small effect on the optical-rotatory-dispersion curve of avidin. 3. The absence of negative circular dichroism at wavelengths above $216\text{m}\mu$ showed that there was little or no α -helix present in avidin. This interpretation was confirmed by Moffitt-Yang plots of the partial rotation due to the peptide bonds in the visible region of the spectrum. The calculated dispersion constants were remarkably similar to those of γ -globulin and suggested the presence of peptide conformations other than α -helix and random coil. 4. The far-ultraviolet spectrum was also similar to that of γ -globulin, the mean extinction coefficient of the peptide chromophore being much lower than the experimental value for a random-coil structure. 5. Streptavidin resembled avidin in showing two positive Cotton effects, but the negative dichroism below $220\text{m}\mu$ suggested the presence of more α -helix than was found in avidin. Formation of the complex with biotin was accompanied by changes in rotation that were rather larger than those observed with avidin.

Spectroscopic studies of the reaction between avidin and biotin (Green, 1963a) showed it to be accompanied by a marked change in the environment of the tryptophan residues of avidin. They became less exposed to solvent when the biotin was bound. It was hoped that further information about this change could be obtained from a study of the ORD† of the protein. Previous measurements (Green, 1963b) had shown combination with biotin to have little effect on $[\alpha]_D$, but it was thought that there might be specific effects in the neighbourhood of the tryptophan absorption bands. However, the effects at $290\text{m}\mu$ proved to be only slightly greater than those at $589\text{m}\mu$.

Although the ORD curves of avidin and the avidin-biotin complex were very similar to each other they were strikingly different from those of any other protein so far studied, in showing Cotton effects accompanied by positive rotation in the neighbourhood of both tryptophan absorption bands. To facilitate the interpretation of these effects, measurements of the circular dichroism

and far-ultraviolet spectra were also made. Since circular dichroism is quantitatively related to ORD it does not in principle provide any additional information. However, it has the advantage that the dichroism is confined to the wavelength region of the optically active absorption band. The resulting bands are usually discrete and the spectra are more readily interpretable than the ORD. The far-ultraviolet absorption of the peptide bond is dependent on its conformational relation to neighbouring peptide bonds, and this has been used to obtain information on the amounts of helical and random-coil structure in a number of proteins (Rosenheck & Doty, 1961; McDiarmid, 1965). Although the absorption is less sensitive than the ORD to changes in conformation the measurements are relatively simple and more accurate. However, interpretation is still hazardous.

The recently discovered biotin-binding protein, streptavidin, isolated by Chalet & Wolf (1964) from culture filtrates of *Streptomyces avidinii*, differs considerably from avidin in its amino acid composition. However, like avidin, each molecule binds four molecules of biotin and the binding is accompanied by changes in absorption spectrum and reactivity of tryptophan similar to those

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† Abbreviation: ORD, optical rotatory dispersion.

observed with avidin from egg white (N. M. Green, unpublished work). The ORD and circular-dichroism curves are therefore included for comparison with those of avidin.

METHODS

The avidin sample was preparation D.31 of Melamed & Green (1963). The concentrations of its solutions were determined from the E_{280} values. Streptavidin was kindly supplied by Dr F. J. Wolf. Measurements of the biotin-binding capacity by spectrophotometric methods (Green, 1963a) confirmed that 1 mole of biotin was bound by 15000g. of protein (Chalet & Wolf, 1964). The value of E_{280} /mg./ml. was 3.4. Synthetic (+)-biotin was supplied by Roche Products Ltd., Welwyn Garden City, Herts. All other reagents used were of analytical grade.

The ORD measurements were made with two different instruments. Preliminary measurements were made with a Rudolph recording spectropolarimeter (model 250/655/850/810-614) equipped with a 150w xenon arc. The rotation measurements above 300m μ , from which a_0 , b_0 , and λ_c were calculated, were also made with this instrument. Measurements below 300m μ were made with a Bendix Polarmatic 62 recording spectropolarimeter in the Laboratory of Professor W. Klyne. To check the validity of measurements in regions of high absorption, solutions of various concentrations and cells of different path lengths between 0.1 and 5cm. were used. All curves shown are averages of duplicate runs.

Circular-dichroism measurements were kindly made by Dr S. Beychok (Department of Biochemistry, Columbia University) with a modified Jouan Dichrograph. The performance of the instrument is described by Beychok (1965) and Beychok & Fasman (1964). The partial rotations $[\phi_K]$, produced by the K th dichroism band were calculated by the procedure of Moskowitz (1960), by assuming gaussian bands and using the Kronig-Kramers transform:

$$[\phi_K] = \frac{2[\theta_K^0]}{\pi^{\frac{1}{2}}} \left(e^{-x^2} \int_0^x e^{x^2} dx - \frac{\Delta_K^0}{2\lambda + \lambda_K^0} \right)$$

where $[\theta_K^0]$ is the molar ellipticity at the maximum of the K th band, Δ_K is the half band-width and x is $(\lambda - \lambda_K^0)/\Delta_K^0$. The values of the integral were obtained from Mathematical Tables (Jahnke & Emde, 1952). The rotational strengths R_K were calculated by using the relation:

$$R_K \sim 0.696 \times 10^{-42} \pi^{\frac{1}{2}} [\theta_K^0]$$

Far-ultraviolet spectra were measured with a suitably modified Beckman DK-2 recording spectrophotometer (Rosenheck & Doty, 1961) in the Laboratory of Dr W. B. Gratzer (Department of Biophysics, King's College, London). A stock solution of avidin (4mg./ml.) was diluted 1:20 with either 0.105M-NaClO₄ or 0.105N-HClO₄ in 1cm. cuvettes. The difference in E_{233} was followed. When there was no further change in ΔE_{233} , showing completion of the dissociation in HClO₄, the far-ultraviolet spectra were determined. A base line was determined with 0.2cm. Suprasil cells (Unicam Instruments, Cambridge) containing solvent (0.5ml. of 0.1M-NaClO₄ or 0.1N-HClO₄). The appropriate avidin solution (0.2ml.) was then added to one

cell and solvent (0.2ml.) to the other by using calibrated Carlsberg pipettes (H. W. Pedersen, Copenhagen, Denmark). The spectrum was measured and then, with avidin in NaClO₄, 5 μ l. of 0.4mm-biotin was added to both reference and sample cells by using a 10 μ l. Hamilton syringe (Shandon Scientific Co., London, N.W. 10) and the spectrum was redetermined. The mean extinction coefficients of the peptide bond under the different conditions were calculated by subtracting the extinction due to the amino acid side chains from the total extinction, by using the data for the extinction coefficients given by McDiarmid (1965) and the amino acid composition given by Melamed & Green (1963). The method of calculation and the assumptions involved are the same as given by Rosenheck & Doty (1961). The absorption of the acetamido groups of *N*-acetylglucosamine was included as one of the terms in the side-chain absorption.

Sedimentation coefficients were determined in a Spinco model E ultracentrifuge at 15° and at 59780 rev./min.

RESULTS

Since one of the main objects of the experiments was to correlate the changes in environment of the tryptophan residues accompanying biotin-binding with changes in optical rotation, the rotation in the ultraviolet region was first investigated. The ORD curves of avidin and the avidin-biotin complex are shown in Fig. 1 (curves *A*). Below 220m μ the results obtained on the Bendix instrument were difficult to reproduce. However, curves obtained with a Cary model 60 spectropolarimeter confirmed the position of the extremum at 220m μ .

Superficial examination of the curves suggested two positive Cotton effects centred at 278 and 228m μ and this interpretation was confirmed by the circular-dichroism measurements presented below. These Cotton effects disappeared when avidin was dissociated into sub-units in 6M-guanidine hydrochloride. In the curve shown (Fig. 1, curve *C*) a trace of an inflexion remains at 280m μ . This was probably caused by the presence of a small amount of undissociated protein since in some experiments it was absent. When $\alpha\lambda^2$ was plotted against α (one-term Drude equation) these results gave a straight line with $\lambda_c = 216m\mu$, typical of the behaviour of a denatured protein or random-coil polypeptide (Urnes & Doty, 1961). With 0.1N-hydrochloric acid, which brings about partial dissociation of the avidin (Green, 1963b, and Fig. 4 below), greatly diminished Cotton effects were observed (Fig. 1, curve *B*). In contrast with the effects of guanidine hydrochloride, formation of the avidin-biotin complex (Fig. 1, curve *A*) had little effect on the rotation, apart from a slight intensification of the 280m μ Cotton effect. The change in the other Cotton effect was only just outside the rather large limits of error of the short-wavelength measurements. Since the effect of binding of biotin was so small, little further work was done on this aspect of the problem and

Fig. 1. ORD curves of avidin and the avidin-biotin complex. Curve *A*: native avidin (0.86 mg./ml.) in 0.05 M-sodium phosphate buffer, pH 6.8, for wavelengths above 240 mμ; below this wavelength the concentration of avidin was 0.118 mg./ml. in water; 1 cm. path length was used throughout; the broken curve is as for the continuous curve *A*, but for the avidin-biotin complex. Curve *B*: avidin in 0.1 N-HCl; above 240 mμ the concentration of protein was 0.88 mg./ml., and below 240 mμ it was 0.19 mg./ml.; 1 cm. path length was used throughout. Curve *C*: avidin after standing for 17 hr. at room temperature in 6 M-guanidine hydrochloride and then dilution to 3 M-guanidine hydrochloride; the protein concentration above 240 mμ was 10 mg./ml., and below 240 mμ it was 1.2 mg./ml.; 0.1 cm. path length was used throughout.

subsequent experiments were devoted to interpreting the two unusual Cotton effects and to estimating the contribution of helical and random-coil conformations of the peptide bonds to the rotation.

Circular dichroism. The curve for avidin (Fig. 2) showed two positive maxima. The short-wave-length band (228 mμ) was symmetrical and was at the same wavelength as the corresponding absorption band of tryptophan, which, in avidin, is located at about 225–226 mμ (Green, 1962). The band at 274 mμ was at a somewhat shorter wavelength than the absorption maximum of avidin (282 mμ). It is clear that there are two positive Cotton effects, confirming the conclusions drawn

Fig. 2. Circular-dichroism spectrum of avidin. The measurements were made in cells of 0.1 cm. and 1 cm. path length. Avidin (0.57 mg./ml.) was dissolved in 0.1 M-sodium phosphate buffer, pH 6.9.

from the ORD curves. The absence of negative dichroism at wavelengths below 220 mμ suggests that there is little or no right-handed α -helix in avidin.

For the purposes of this section, it will be assumed that both effects originate mainly from the absorption bands of tryptophan. The arguments for this point of view are presented below. The circular-dichroism results shown were based on a mean residue weight of 119 (Melamed & Green, 1963). However, if the dichroism is mainly caused by tryptophan residues it would be more informative to calculate it per mole of tryptophan. The molar ellipticity $[\theta]_{228}$ becomes $+100\,000\text{ cm}^2/\text{decimole}$ (the rotational strength $R_{228} = 46 \times 10^{-40}$ c.g.s. units) and $[\theta]_{274}$ becomes $+11\,500\text{ cm}^2/\text{decimole}$ ($R_{274} = 9.3 \times 10^{-40}$ c.g.s. units). These values are about one order of magnitude greater than those for the Cotton effects of polytyrosine (Beychok & Fasman, 1964) or free tryptophan (Legrand & Viennet, 1965). Similar calculations may be applied to the ORD Cotton effects, the values of $[M]$ being multiplied by 31 to express them as rotations per mole of tryptophan. The amplitudes of the two effects $[\Delta M]$ become $150\,000^\circ$ and $13\,000^\circ$ for the 228 mμ and 274 mμ regions respectively. These values are less reliable than the

dichroism results as a guide to the rotational contribution of tryptophan since the peptide bonds contribute far more to the rotation than to the dichroism in this region.

Optical rotatory dispersion in the visible region. It is now possible to make a more meaningful interpretation of the ORD data from the visible region of the spectrum. These are plotted according to the Moffitt–Yang equation (Urnes & Doty, 1961) in Fig. 3. The dispersion constants calculated from these plots are given in Table 1. Although this procedure is not strictly valid for native avidin, on account of the tryptophan Cotton effects, linear relationships were observed down to $400m\mu$. Avidin dissociated into sub-units in 3M-guanidine hydrochloride gave $a_0 = -480$ and $b_0 = 0$, as would be expected for a denatured protein (Urnes & Doty, 1961). However, native avidin in either water or 3M-guanidine hydrochloride was unusual in giving a positive b_0 . Both this and the departure of the experimental points from the straight line at shorter wavelengths (Fig. 3) were probably caused by the tryptophan Cotton effects. The partial rotations contributed by these effects were therefore calculated from the circular dichroism by using the Kronig–Kramers transform (Moskowitz, 1960) and subtracted from the total rotation. The resulting values should approximate more closely to the true partial rotation of the peptide bond, although it should be remembered that they will include contributions from aromatic and other side chains with absorption bands in the far ultraviolet. The Moffitt–Yang plot (Fig. 3) was linear down to $313m\mu$ and b_0 was now much lower (+67). This result confirms the conclusion from the dichroism

measurements that there is little or no right-handed α -helix in avidin.

Far-ultraviolet absorption. The absorption spectra of avidin in 0.1M-sodium perchlorate, with and without biotin, and in 0.1N-perchloric acid are shown in Fig. 5. Perchloric acid, which is transparent below $200m\mu$, was used in place of guanidine hydrochloride to bring about partial dissociation of the avidin into sub-units. The extent of dissociation had previously been determined in 0.1N-hydrochloric acid by sedimentation experiments (Fig. 4), which show that most of the avidin was present as sub-units ($S_{20,w}$ 2.1s). The avidin–biotin complex was not affected by the low pH.

The differences between the three spectra were very small. The presence of biotin had a negligible effect (less than 1%) below $225m\mu$. Even in acid, where only about 30% of the $228m\mu$ Cotton effect remained (Fig. 1), there was only a slight increase in the extinction. The shoulder at $220m\mu$ is due to tryptophan and the shifts in the $230m\mu$ region are in agreement with previously published data on the difference spectra (Green, 1963a,b). The mean extinction coefficients of the peptide bonds were calculated by subtracting the contribution of the amino acid side chains. They are shown in Fig. 5 in relation to the experimental values for α -helical and random-coil conformations of synthetic polypeptides (Rosenheck & Doty, 1961). If the data are interpreted as suggested by Rosenheck & Doty (1961) one would conclude that between 60 and 100% of the peptide bonds in avidin were in α -helical conformation and that even in acid, where most of the aromatic Cotton effect has disappeared, about 60% of this conformation remains intact. Such conclusions clearly conflict with the

Fig. 3. Moffitt–Yang plots of ORD data of avidin. The measurements were made in 5cm. cells on solutions of avidin (8mg./ml.) in 0.2M-sodium phosphate buffer, pH 6.8. ●, Avidin; ▲, avidin after subtraction of the aromatic contribution, as described in the text; ■, avidin, dissociated into sub-units in guanidine hydrochloride (6M); the guanidine hydrochloride concentration was diluted to 3M before the ORD was measured.

Fig. 4. Sedimentation of avidin and the avidin–biotin complex in 0.1N-HCl. Upper curves: avidin (8mg./ml. in 0.1N-HCl), $S_{20,w}$ of fast component 4.55s and that of slow component 2.15s. Lower curves: avidin–biotin complex (7.4mg./ml. in 0.1N-HCl), $S_{20,w}$ 4.55s. Photographs were taken after (a) 76 min. and (b) 140 min.

Fig. 5. Ultraviolet spectra of avidin and the avidin-biotin complex. The upper pair of curves show the observed extinction coefficients, expressed as an average value per peptide bond. The lower pair of curves show the extinction coefficients after subtraction of the contributions of the amino acid side chains. The upper reference lines at 190, 197 and 205 $m\mu$ give the extinction coefficients of the peptide bond in a random-coil polypeptide, and the lower ones give the corresponding values for an α -helix (Rosenheck & Doty, 1961). —, Avidin and avidin-biotin complex in 0.1M-NaClO₄; ····, avidin-biotin complex, when distinguishable from avidin; - - - -, avidin in 0.1N-HClO₄.

results of the ORD measurements. The discrepancy is discussed further below.

Near-ultraviolet absorption of avidin. It has been suggested (Fasman, Bodenheimer & Lindblow, 1964; Fasman, Landsberg & Buchwald, 1965) that the Cotton effects of the aromatic residues in polytyrosine and polytryptophan may result from electronic interaction between neighbouring residues. A further possible consequence of such interactions would be a decreased E_{280} value. The possibility that this might be so in avidin was checked by following E_{280} when avidin was allowed to stand in 6M-guanidine hydrochloride. A 4.0% decrease in E_{280} was observed. This small effect is similar in magnitude and direction to that observed on denaturation of many proteins. It can most readily be explained in terms of an effect of solvent (in this case the protein environment) on the extinction coefficients of the individual tryptophan residues. For example, the extinction coefficient of acetyltryptophan ethyl ester is increased by 10% on transfer from water to ethanol (G. H. Beaven, personal communication).

Streptavidin. The ORD curves of streptavidin and its complex with biotin (Fig. 6) show similar positive Cotton effects in the neighbourhood of the two tryptophan absorption bands. The shorter-wavelength effect is somewhat further to the red

Fig. 6. ORD curves of streptavidin and the streptavidin-biotin complex. The streptavidin (0.31mg./ml.) was dissolved in 0.1M-NH₄ClO₄, pH 8.0, for measurements down to 240 $m\mu$. Below this wavelength the solution was diluted to 0.045mg./ml. All measurements were made in cells of 1cm. path length. The left-hand ordinate scale refers to measurements below 233 $m\mu$, and the right-hand scale to measurements at higher wavelengths. —, Streptavidin; - - - -, streptavidin-biotin complex.

than with avidin and the negative extremum is much more marked.

The effect of combination with biotin on the short-wavelength Cotton effect is somewhat greater than with avidin, the rotation at the positive extremum (232 $m\mu$) becoming considerably more negative. As with avidin (Fig. 2), the circular dichroism of streptavidin (Fig. 7) shows two positive bands, though the shorter-wavelength one is shifted to the red relative to that in avidin. In contrast with avidin the dichroism becomes negative below 225 $m\mu$.

DISCUSSION

The small effect of biotin on the ORD curve of avidin does not add any information to that already available from other spectroscopic studies, except in the negative sense that it excludes any change in total amount of α -helix consequent on the binding of biotin. The slight increase in

amplitude of the 279m μ Cotton effect, like the red shift of the absorption spectrum, could be caused by an increase in the refractive index of the medium surrounding the tryptophan. The effect of biotin on the ORD of streptavidin is somewhat greater and is considered below.

The results provide no evidence for any right-handed α -helix in avidin. There was no sign of any negative contribution to the dichroism in the 220m μ region, and, after correction for the contribution of the tryptophan Cotton effects, b_0 was almost zero. In spite of the low value of b_0 , it is difficult to reconcile the results with a predominantly random arrangement of peptide bands. Randomly coiled polypeptides show rotations of about -3000° in the 230m μ region and there is no sign of such a strong negative effect in the

avidin ORD curve. Both the Cotton effects are centred on a flat, almost zero, background and not on a rapidly descending negative one. It appears that other influences must be present that in part neutralize the negative circular-dichroism band of the random coil in the far ultraviolet (Holzwarth & Doty, 1965). At least two factors may operate in this fashion: (1) the absorption band of tryptophan in the far ultraviolet (ϵ_{193} 20000); (2) the arrangement of some of the peptide bonds in a β -conformation (Urnes & Doty, 1961; Imahori, 1963). It is also possible that there is very little random coil present and that most of the peptide bonds are in either a left- or a right-handed helical conformation. The absorption band of tryptophan in the far ultraviolet almost certainly makes some contribution to the rotation, though it is unlikely to be large enough to counteract a random-coil peptide contribution. The evidence for the presence of β -structure in globular proteins is slight, but Imahori (1963), Callaghan & Martin (1963), Martin (1965) and Sarkar & Doty (1966) have produced some evidence for its presence in γ -globulin. Table 1 shows that the dispersion constants a_0 , b_0 and λ_c calculated for the partial rotation of the peptide bonds of avidin are remarkably similar to those of γ -globulin (Gould *et al.* 1964), which in turn differ markedly from those of most other proteins (Urnes & Doty, 1961). The far-ultraviolet spectrum provides a further point of resemblance between avidin and γ -globulin, since both proteins have mean peptide extinction coefficients that suggest a high proportion of helical structure in the molecule (Rosenheck & Doty, 1961), in conflict with the evidence from their ORD.

There are, however, two observations that are difficult to reconcile with the presence of any large proportion of β -structure. First, neither avidin (N. M. Green, unpublished work) nor γ -globulin (Gould *et al.* 1964) shows any sign of a 1610cm. $^{-1}$ shoulder in the infrared spectrum, which would be characteristic of the amide I absorption of peptide bonds in this conformation. Secondly, the extinction coefficient of the peptide bond in a β -structure (ϵ_{197} 7500) is higher than in random conformation (ϵ_{197} 6300) (Rosenheck & Doty, 1961), whereas

Fig. 7. Circular-dichroism spectrum of streptavidin. The measurements were performed in cells of 1 cm. of 0.1 cm. path length in 0.05M-NH₄Cl adjusted to pH 9.0 with aq. NH₃. The streptavidin concentration was 0.05 mg./ml.

Table 1. Dispersion constants of avidin compared with those of rabbit γ -globulin

	$[\alpha]_D$	λ_c	a_0	b_0
Avidin	-19.8°	—	-148	146
Avidin after subtraction of aromatic contribution	-50.5	190	-325	67
Avidin in guanidine hydrochloride (3M)	-76.1	210	-480	4
γ -Globulin* (rabbit) (pH 7.3)	-58	195	-275	35
γ -Globulin* (rabbit) (pH 1.9)	-76	—	-350	10

* Gould, Gill & Doty (1964). Further data on human γ -globulin and Bence-Jones proteins are given by Urnes & Doty (1961) and Callaghan & Martin (1963).

avidin (ϵ_{197} 4800) and γ -globulin (ϵ_{197} 5400) both show values that are unusually low for proteins containing little α -helix. The presence of equivalent amounts of left- and right-handed helix in γ -globulin is unlikely, since the peptide hydrogen atoms exchange rapidly. One cannot exclude this possibility in avidin, though it seems an unlikely one.

Aromatic contribution to the Cotton effects. In view of the coincidence of the dichroism bands of avidin with the absorption bands of tryptophan they can be assigned to this chromophore with some certainty. The shift of the long-wavelength maximum to 274m μ (avidin) or 270m μ (streptavidin) appears to be within the normal range, since Legrand & Viennet (1965) have observed the corresponding band of free tryptophan to be at 266m μ . Small contributions from tyrosine, which is responsible for 5% of the E_{280} value, and cystine (Beychok, 1965) cannot be excluded. A left-handed α -helix would produce positive dichroism in the 230m μ region, but by analogy with the right-handed helix (Holzwarth & Doty, 1965) one would expect the band to be much broader and to be located at shorter wavelengths than was in fact observed with avidin. However, this possibility should be kept in mind. The results given by streptavidin are consistent with the presence of a small amount of right-handed α -helix, whose negative dichroism peak would be at 219m μ (Holzwarth & Doty, 1965) and would partially cancel the positive dichroism caused by tryptophan. This would account for the dichroism of streptavidin becoming negative below 225m μ , and for the asymmetry of the 232m μ band. Similarly, it would explain the differences between the ORD curves of avidin and streptavidin. Both the strong negative extremum of the streptavidin ORD at 217m μ and the apparent red shift of the positive extremum to 238m μ could be explained by the presence of two overlapping Cotton effects of opposite sign. The rotation at 238m μ was much more affected by biotin than was that of avidin, and its direction suggested a small increase in the amount of α -helix. This would have little effect in the 280m μ region, where the ORD curves for helix and coil lie close together (Blout, Schmier & Simmons, 1962).

Several reports of Cotton effects caused by tyrosine and tryptophan in proteins have appeared. The earliest examples were small inflexions in the 280m μ region shown by tobacco-mosaic virus (Simmons & Blout, 1960) and carboxypeptidase (Fujioka & Imahori, 1962). Larger effects have been observed with carbonic anhydrase (Myers & Edsall, 1965), lysozyme (Glazer & Simmons, 1965) and cytochrome *c* (Urry & Doty, 1965). The amplitude of the effects at 290m μ in lysozyme and

carbonic anhydrase was about 4000°/mole of tryptophan (avidin 13000°). So far no other protein has shown a positive Cotton effect in the 230m μ region, presumably because any such effect would be masked by the much stronger negative Cotton effect produced by a small proportion of right-handed α -helix. It is the absence of α -helical arrangement of peptide bonds that causes the two avidin effects to stand out so clearly. Streptavidin provides an intermediate case in which the two opposing effects are of similar magnitude, but, being centred at slightly different wavelengths, only partially cancel each other.

In all these examples the effects disappear when the protein is denatured, showing that much of the dissymmetry is provided by the tertiary structure of the protein, though not by α -helix in avidin. This is not surprising in view of the Cotton effects observed in the visible region when many symmetrical chromophores are bound to proteins (cf. Ulmer & Vallee, 1965); indeed, it is the weakness or absence of aromatic Cotton effects in proteins that is remarkable. Tyrosine and tryptophan are hydrophobic residues, and are likely to be situated in the interior of the protein in a rigid and asymmetric environment. It is possible that when several such residues are present their partial rotations cancel, but this is unlikely to be general. The most likely alternative hypothesis is that only certain types of asymmetric perturbation can produce strong Cotton effects. Fasman *et al.* (1964, 1965) studied the ORD of solutions and films of polytryptophan, polytyrosine and of their copolymers with glutamic acid. Tyrosine copolymers showed Cotton effects only when they contained more than 20% of tyrosine. Fasman *et al.* (1964, 1965) suggested that this was because the Cotton effects were dependent on tyrosine-tyrosine interactions. Similar interactions between tryptophan residues were invoked to explain the multiple Cotton effects observed in the 280m μ region and the 10% hypochromism of polytryptophan. [This latter effect was not observed by Shifrin (1961) with polytryptophan samples of lower molecular weight.] If strong tryptophan-dependent Cotton effects require interaction between two or more closely situated residues this could explain their rarity. However, such interactions could lead both to hypochromism and to a splitting of the aromatic dichroism bands into two components of opposite sign (cf. Tinoco, 1964; Van Holde, Brahms & Michelson, 1965). There is no indication of such a splitting of the dichroism bands of avidin, nor did the slight decrease in E_{280} accompanying the dissociation of avidin into sub-units support this hypothesis. Although the present state of theory is such that one cannot exclude electronic interactions between tryptophan residues on these

grounds, it seems more likely that the Cotton effects are due to some other perturbation of the aromatic chromophore.

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