A Spin Label Study of Egg White Avidin

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Avidin is a tetrameric protein (mass 68,000 daltons) that binds 4 molecules of vitamin biotin (I). The biotin binding sites, 1 per subunit, are grouped in two pairs at opposite ends of the avidin molecule (Green, N. M., Konieczny, L., Tomes, E. J., and Valentine, R. C. (1971) Biochem. J. 125, 781). We have studied the topography of the avidin binding sites with the aid of four spin-labeled analogs of biotin: 4-biotinamido-2,2,6,6-tetramethyl-1-piperidinyloxy (II), 3-biotinamido-2,2,5,5-tetramethyl-1-pyrroldinyloxy (III), 3-biotinamidomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (IV), 4-(biotinylglycyl)-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (V). Fluorescence and optical absorption spectroscopy indicated that II to V occupied the same binding sites on avidin as did biotin. The electron spin resonance spectrum of the 4:1 complex between II and avidin contained broad line components characteristic of a highly immobilized spin label. Dipole-dipole interactions between spin labels bound to adjacent sites split each of the three major hyperfine lines into doublets with a separation of 13.8 G. The distance between adjacent bound nitroxide groups was calculated from this splitting to be 16 Å. The dissociation of the 4:1 complex between II and avidin was biphasic with approximately half of the labels dissociating at a rate (k_{diss} = 2.51 \times 10^{-4} \text{ s}^{-1}) that was much faster than the remainder (k_{diss} = 1.22 \times 10^{-5} \text{ s}^{-1}). The electron spin resonance spectrum of the 2:1 complex between II and avidin clearly showed that, immediately after mixing, the spin labels were distributed in a random fashion among the available binding sites but that they slowly redistributed themselves so that each label bound to a site which was adjacent to an unoccupied site. The final time-independent electron spin resonance spectrum exhibited a splitting of 69 G between the low and high field hyperfine lines which is characteristic of a highly immobilized, noninteracting spin label. Spin labels III and IV interacted with avidin in a similar fashion to that described for II with the exception that their dipolar splittings were 11.9 G and 14.2 G, respectively. From these splittings it was estimated that the distance between adjacent avidin-bound nitroxides was 16.7 Å for label III and 15.7 Å for label IV. The electron spin resonance spectrum of label V bound to avidin was characteristic of a noninteracting highly immobilized nitroxide with a maximum splitting of 62 G. The spectrum of V bound to avidin was independent of both time and the amount of bound label. The rate of dissociation of V from a 4:1 complex with avidin was monophasic. A model is proposed in which the recognition site for the heterocyclic ring system of biotin is represented as a cleft located within a hydrophobic depression in the surface of avidin.

Avidin is a tetrameric protein (mass 68,000 daltons), found in raw egg white, which binds 4 molecules of biotin (I) (vitamin H) (1–3) (Scheme 1). Green et al. have studied the location and spatial relationship of the biotin binding sites on avidin by means of a series of bis-biotinyl diamines in which the carboxyl groups of the 2 biotin residues were separated by chains containing between 9 and 25 bonds (4). These workers found that, when there were fewer than 12 bonds between the carboxyl groups of the 2 biotin residues, they were able to form intermolecular bridges and gave rise to linear polymers of avidin. Compounds with longer chains were able to form intra- and intermolecular bridges and gave rise to linear polymers of avidin. Polymers formed with reagents that contained connecting chains with 12, 13, or 14 bonds were relatively unstable and could be depolymerized by weakly bound analogs of biotin. However, polymers formed with the longer chain reagents were not depolymerized under these conditions.

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Scheme 1

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was a cavity in a quartz micro cell (Varian E-248-1) while the temperature activation and emission were 12 nm. The spectrometer operating at 9.5 GHz. Quartz aqueous sample cells were used. The activating wavelength was 290 nm while the bandwidths of the labels were calculated from the areas under their fluorescence emission curves. The relative tryptophan fluorescence of avidin was estimated either from the absorption of the solution at 280 nm (\(E_{m}^{\text{av}} = 15.4\)) (9) or by titration with HABA according to the procedure of Green and Toms (10). The concentration of avidin was 1 mg/ml (14.7 \(\mu\)M), the concentration of biotin was 200 \(\mu\)g/ml, and the buffer was 50 mM sodium phosphate, pH 7.4.

**EXPERIMENTAL PROCEDURES**

**Materials—** Avidin was purchased from Worthington Biochemical Corp. and purified by chromatography on carboxymethyl-cellulose according to the procedure of Green and Toms (9). The concentration of avidin was estimated either from the absorption of the solution at 280 nm (\(E_{m}^{\text{av}} = 15.4\)) (9) or by titration with HABA according to the procedure of Green (10). d-Biotin and HABA were purchased from Sigma Chemical Co. All other chemicals were of reagent grade. The molar extinction coefficients for free and avidin-bound biotin were synthesized by previously reported procedures. A detailed account of their syntheses will appear elsewhere.2

**Methods—** Absorption measurements were made with a Beckman model DU spectrophotometer using 10-mm path length quartz cells. The displacement of HABA from avidin by biotin and the spin labels was measured by monitoring the change in absorption of the dye at 500 nm (10). The molar extinction coefficients for free and avidin-bound HABA at this wavelength are 600 and 3450, respectively (10).

Fluorescence measurements were made at 25° with 10-mm2 quartz cells in an Aminco-Bowman spectrophotofluorometer equipped with an off-axis ellipsoidal mirror, a ratio recording attachment, and a R106 photomultiplier tube. The emission grating of the spectrophotofluorometer was blazed at 300 nm so that corrected emission spectra were obtained over the range 300 to 450 nm (11). The relative tryptophan fluorescence quantum yields of avidin and its complexes with biotin and the spin labels were calculated from the areas under their fluorescence emission curves. The activating wavelength was 290 nm while the bandwidths of activation and emission were 12 nm.

Electron spin resonance spectra were recorded with a Varian E-4 spectrometer operating at 9.5 GHz. Quartz aqueous sample cells (Varian E-248) were used to obtain spectra at ambient temperature (25°). For all other temperatures, the samples were introduced into the cavity in a quartz micro cell (Varian E-248-1) while the temperature was maintained with a Varian E-257 variable temperature accessory. Temperatures were measured with a Yellow Springs Instrument Co. telethermometer model 42SC. The subtraction of ESR spectra was achieved by first collecting the spectra in a Nicolet Instrument Corp., model 1072 computer, punching them onto paper tape, and then reading the tape into a Digital Equipment Corp. PDP10 computer. The spectra were then manipulated in the form of matrices using the MLAB program (12). A Calcomp plotter was used to obtain a hard copy of the subtracted spectra.

The sedimentation coefficients of avidin and its 4:1 complex with spin label II were measured in a Beckman model E ultracentrifuge. The concentration of avidin was 1 mg/ml (14.7 \(\mu\)M), the concentration of spin label II, when present, was 59.0 \(\mu\)M and the buffer was 50 mM sodium phosphate, pH 7.4.

**RESULTS**

In order to establish that the biotin spin labels were binding to the same site on avidin as biotin itself, their interaction with avidin was studied by three independent spectroscopic techniques: (a) displacement of HABA, (b) the change in the absorption of avidin at 233 nm, and (c) the quenching of avidin tryptophan fluorescence. Green has shown (10) that when HABA binds to avidin the absorption maximum of the dye shifts from 348 to 480 nm. Each avidin molecule has four binding sites for HABA from which the dye can be displaced by biotin. The addition of spin label II to the avidin complex resulted in the displacement of an equivalent amount of dye (Fig. 1). Similar results were obtained with labels III to V. The binding of biotin and its analogs to avidin is accompanied by an increase in the optical density of the protein at 233 nm (8). When increments of spin label II were added to avidin, the optical density of the protein at 233 nm increased in a linear fashion until 1 eq of label had been added per biotin binding site (Fig. 2). Similar results were obtained with the other spin labels. Green has reported that the binding of biotin to avidin produces a shift in the tryptophan fluorescence emission maximum of the protein to shorter wavelengths and a decrease in the quantum yield of avidin tryptophan fluorescence (13). In our experiments the fluorescence emission maximum of avidin shifted from 336 to 326 nm on the addition of 4 eq of biotin. The binding of biotin to avidin quenched the tryptophan fluorescence quantum yield of the protein by 31%. This value compares well with the 30% quenching of avidin fluorescence by biotin reported by Green (13). The relative fluorescence quantum yields and emission maxima of complexes between avidin and the biotin spin labels are shown in Table I. The fluorescence titration curves of biotin was labeled biotin analogs with avidin.

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Titration of a mixture containing avidin (5 \(\mu\)M) and HABA (90 \(\mu\)M) in 50 mM sodium phosphate buffer, pH 7.4, with spin label II.
and the four spin labels showed sharp breaks when 1 eq of the ligands had been added per binding site (Fig. 3). These experiments, which are based on three different spectroscopic techniques, demonstrate quite clearly that the spin labels occupy the same sites on avidin as does biotin itself.

The ESR spectrum of a dilute solution of spin label II in sodium phosphate buffer (50 mM, pH 7.4) consisted of three sharp lines of about the same intensity with a splitting of 17 G between adjacent hyperfine lines. The ESR spectrum of a 2:1 molar complex of spin label II with avidin contained broad line components and was time-dependent (Fig. 4). The final time-independent spectrum (Figs. 4G and 5A) was that of a highly immobilized nitroxide radical with a splitting of 69 G between the low and high field extrema. Superposition of spectra obtained at successive time intervals after mixing revealed the presence of several well defined isoclinic points (Fig. 4). Isoclinic points in first derivative spectra must be interpreted with caution, particularly when they occur at or near the base-line. However, Marriott and Griffith have shown (14) that when multiple isoclinic points occur in regions of the first derivative ESR spectra of nitroxides that are away from the base-line then there is a high probability that isosbestic points will be found in the corresponding absorption spectra. Thus it seems reasonable to assume that the spectra shown in Fig. 4 are derived from two species of bound spin label II, one of which is slowly converted into the other. Double integration of the initial and final spectra (Fig. 4, A and G) indicated that there was no loss of spin intensity during the experiment. Spectra similar to those shown in Fig. 4 were also obtained when less than 2 eq of spin label II were added to avidin. When additional increments of II were added to the time-independent 2:1 complex, the ESR spectrum of the complex began to broaden further, new peaks appeared at the low and midfield

### Table I

**Fluorescence emission maxima and relative quantum yields of complexes between avidin and biotin and biotin spin labels**

Relative quantum yields were measured as described under “Methods.” The activation wavelength was 290 nm, while the bandwidths for activation and emission were 12 nm. The following concentrations were employed: avidin, 5.5 μM; sodium phosphate buffer (pH 7.4), 50 mM; and, where indicated, biotin and the biotin spin labels, 22.0 μM.

| Compound              | Emission maximum | Relative quantum yield |
|-----------------------|------------------|------------------------|
| Avidin                | nM               | 100                    |
| Avidin + biotin       | 326              | 68.8                   |
| Avidin + II           | 326              | 18.2                   |
| Avidin + III          | 325              | 16.0                   |
| Avidin + IV           | 335              | 39.3                   |
| Avidin + V            | 330              | 62.4                   |

![Fig. 2. Titration of avidin (4.1 μM) in 50 mM sodium phosphate (pH 7.4) with biotin (△—△) or spin label II (∙—•).](http://www.jbc.org/)

![Fig. 3. Titration of avidin (5.5 μM) in 50 mM sodium phosphate buffer (pH 7.4) with spin labels II (△—△), III (△—△), IV (△—△), V (■—■), and biotin (∙—•).](http://www.jbc.org/)

![Fig. 4. The ESR spectrum of a solution containing spin label II (0.12 mM) and avidin (69.0 μM) in 50 mM sodium phosphate buffer (pH 7.4). Spectra were recorded at the following times after mixing: A, 3.5 min; B, 11.5 min; C, 20.5 min; D, 30.5 min; E, 46.5 min; F, 75.5 min; G, 156.5 min. Scan time was 4 min.](http://www.jbc.org/)

![Fig. 5. The ESR spectrum of spin label II bound to avidin (80.0 μM) in 50 mM sodium phosphate buffer (pH 7.4). The concentration of spin label was 0.11 mM in A and 0.32 mM in B. The arrows mark the position of the sharp three-line spectrum of a small amount of unbound spin label. Spectrum A was recorded 3 hours after the addition of spin label II.](http://www.jbc.org/)
positions and the high field peak became asymmetric (Fig. 5B). Unbound spin label appeared in the spectrum only after 4 mol of spin label II had been added per mol of avidin.

Complexes between spin labels III or IV and avidin, that contained 2 eq or less of bound label, showed the same time-dependent spectral change observed with label II (Fig. 4). The time-independent ESR spectra of 1:1 complexes between label III (Fig. 6) or IV (Fig. 7) and avidin exhibited maximal splittings of 64.9 G and 63.5 G, respectively. The ESR spectra of 4:1 complexes of III (Fig. 6) and IV (Fig. 7) with avidin also contained broad line components which were absent in the 1:1 complexes. The ESR spectrum of spin label V bound to avidin was that of a highly immobilized, noninteracting spin label with a splitting of 62 G between the high and low field extrema (Fig. 8). The spectrum was time-independent and was the same for all ratios of bound spin label to avidin.

When the 4:1 complex between spin label II and avidin was heated, little change was observed in its ESR spectrum at temperatures below 40°. Above 40°, however, the splitting between the broad lines of the low field doublet decreased until at 80° they coalesced to form a single peak (Fig. 9). Over this same temperature range, the low field line of the center doublet became more prominent and moved upfield, while the high field doublet moved downfield and became more symmetrical. The splitting between the low and high field extrema was 60 G at 80°. These changes were not due to irreversible heat denaturation of the avidin, since they were completely reversed upon cooling. In contrast to the 4:1 complex, the 2:1 complex showed no striking change in over-all line shape on heating from room temperature to 60°, although the maximum splitting did decrease from 69 G at 25° to 62.5 G at 60°. It proved impossible to make measurements at temperatures above 60° with the 2:1 complex, because avidin was irreversibly denatured by such treatment.

No change in the ESR spectrum of the 4:1 complex between spin label II and avidin was observed from pH 3 to pH 10. However, in the presence of 0.5 N KOH, the high and low field doublets of the 4:1 complex became more clearly resolved (Fig. 10). This spectral change was completely reversible so that upon neutralization the ESR spectrum returned to that shown in Fig. 5B. No alteration in the ESR spectrum of the 4:1 complex was observed on the addition of 1 M NaCl which...
suggested that the spectral changes induced by 0.5 N KOH were not due to an increase in the ionic strength of the solution. The ESR spectrum of the time-independent 2:1 complex was unaffected by 0.5 N KOH.

If the biotin binding sites on avidin are arranged in the fashion described by Green et al. (4), then it seems reasonable to assume that in the 4:1 complexes between the protein and the spin labels the nitroxide groups might be located sufficiently close to one another for nitroxide-nitroxide interactions to occur. An examination of the ESR spectra of the 4:1 complexes between spin labels II to IV and avidin (Figs. 5 to 7) clearly indicates that some kind of nitroxide-nitroxide interaction is taking place. By contrast, the spectrum of the 4:1 complex between label V and avidin is that of a highly immobilized noninteracting nitroxide radical (Fig. 8). Ferruti et al. have synthesized a series of dinitroxides and have recorded their ESR spectra over a range of temperatures in solvents of widely differing viscosities (15). During the course of their rather extensive study, they observed spectra which could be attributed to: (a) dinitroxides in which there was no interaction between the adjacent radicals, (b) dinitroxides in which there was spin exchange, (c) dinitroxides in which dipolar interactions were present, and (d) dinitroxides in which more than one kind of interaction was occurring. For any given dinitroxide, the predominant interaction was dependent both upon its conformation and its tumbling rate. Ferruti et al. found that spin exchange was encountered most frequently when the dinitroxides were dissolved in solvents with low viscosities (15). Under these conditions, the molecular motion of the dinitroxides was sufficient to bring the adjacent free radicals into close enough proximity to permit the direct electronic overlap that is necessary for this kind of interaction. These workers also found that gradual freezing of dinitroxide solutions gave rise to three types of biradical spectra: (a) those that showed no pairwise interaction, (b) dipolar split ones, and (c) those in which both types of interaction were evident.

When molecular motion is essentially absent, dipolar interactions will split each of the hyperfine lines of the nitroxide into two components. The over-all shape of the resultant spectrum will depend upon the relationship between $|2D|$, the maximal splitting of the peaks in gauss, and $2A_{zz}$, where $A_{zz}$ is the $z$-component of the hyperfine splitting tensor (5, 16). A comparison of the spectra in Fig. 5, A and B, shows that, in the 4:1 complex, each of the hyperfine lines found in the time-independent 2:1 spectrum has been split into a doublet. This splitting is more clearly seen in the spectrum of the 4:1 complex of II and avidin in 0.5 N KOH (Fig. 10), where the asymmetry of the high field line is more apparent. Clearly, for spin label II bound to avidin, we have a situation in which $|2D| < 2A_{zz}$. While it is obvious that dipolar interactions are also taking place in the 4:1 complexes between labels III or IV and avidin (Figs. 6 and 7), it is also apparent that some other complicating factor is present. A closer examination of the spectrum of the 4:1 complex between label IV and avidin reveals the presence of at least two components (see arrows 1 and 2 in Fig. 7B) which could be attributed to a noninteracting, highly immobilized nitroxide. It will be recalled that Ferruti et al. also reported that some of their biradicals in frozen solution contained contributions from both noninteracting and dipolar split nitroxides (15). The spectrum of the 4:1 complex between label III and avidin also contains at least one component (see arrow 1 in Fig. 6B) in the low field region which could be due to a noninteracting nitroxide. No direct spectral evidence exists for the presence of noninteracting nitroxide in the 4:1 complex between spin label II and avidin (Fig. 5B). However, if the assumption is made that noninteracting spin label does contribute to the spectrum, then the spectral change observed on going from sodium phosphate buffer (pH 7.4) (Fig. 5B) to 0.5 N KOH (Fig. 10) could be explained in terms of a shift in the population of bound spin labels from noninteracting to dipolar split nitroxides.

If the assumption is made that the ESR spectra of 4:1 complexes between spin labels II to IV and avidin contain contributions from noninteracting nitroxides, then it should be possible to obtain the dipolar split spectra by spectral subtraction. Increments of the time-independent spectrum of the 2:1 complex between label II and avidin (Fig. 5A) were therefore subtracted from the spectrum of the 4:1 complex (Fig. 5B) with the aid of a computer. Subtraction was continued until a coherent spectrum emerged in which the amplitude of the high field line of the hyperfine doublet was maximal and/or the dipolar splittings of the low and middle field doublets were identical. The computer subtracted spectrum, obtained on the assumption that 30% of the spin labels in the 4:1 complex were noninteracting, is shown in Fig. 11. If the assumption was made that either 25 or 35% of the spectrum shown in Fig. 5B was attributable to noninteracting labels, the subtracted spectrum was still very similar to that shown in Fig. 12. Similar subtractions from the 4:1 spectra of labels III and IV produced the spectra shown in Figs. 12 and 13. It is entirely possible that other interactions are occurring in the 4:1 complexes between spin labels II to IV and avidin. However, the emergence of

Fig. 10. The ESR spectrum of spin label II (0.1 mM) and avidin (25 μM) in 0.5 N KOH.

Fig. 11. The ESR spectrum of spin label II bound to avidin derived from Fig. 5B by computer subtraction of Fig. 5A.
coherent dipolar split spectra leads us to believe that, if present, such interactions make little contribution to the ESR spectra of the 4:1 complexes.

One possible explanation for the presence of noninteracting spin labels in the 4:1 complex of spin labels II to IV is that some of the avidin tetramers dissociate to form monomers when all four sites were occupied by the labels. This seems unlikely, because Green has shown that, in dilute buffer at neutral pH, the subunits of avidin do not dissociate (2). Furthermore, the biotin-avidin complex does not dissociate in either 6 M guanidine hydrochloride or 8 M urea (2). Nevertheless, several attempts were made to demonstrate the presence of monomers in solutions containing the 4:1 complex of spin label II and avidin. When the complex was passed through Sephadex G-150, it emerged as a single symmetrical peak with the same void volume as avidin alone. From analytical ultracentrifugation experiments, the sedimentation coefficients for avidin and its 4:1 complex with spin label II were calculated to be 4.24 S and 4.29 S, respectively. Finally, dilution (1:100) of a solution containing avidin (1 × 10⁻⁴ M) and spin label II (4 × 10⁻⁴ M), which was expected to shift the equilibrium in favor of the monomer, had no effect on the ESR spectrum of the complex. All these findings strongly suggest that the 4:1 complex of avidin and II does not dissociate into monomers. It is also possible that our avidin contained some bound biotin, which was not removed during purification. However, a sample of avidin kindly supplied by Dr. N. M. Green exhibited the same 4:1 spectrum as that shown in Fig. 5B.

An examination of Fig. 4 reveals that, although some of the bound spin labels are undergoing dipolar interactions immediately after mixing, no dipolar interactions are present 2½ hours later. These results suggest that, on mixing, some of the spin labels are bound to sites that are adjacent to sites occupied by other labels, while the remainder of the labels are bound to sites that are adjacent to empty sites. After mixing, the spin labels must rearrange in such a fashion that each is bound to a site which is adjacent to an empty site. Two possible mechanisms can be proposed for such a rearrangement: (a) that the subunits dissociate and then reassociate in such a way that each bound spin label is adjacent to an empty site or (b) that spin labels bound to sites adjacent to occupied sites dissociate more readily and then rebind to sites adjacent to empty sites. The former explanation is unlikely in the light of previously discussed work by Green (2). In order to test the second mechanism, the rate of dissociation of spin label II from the 4:1 complex was measured after the addition of a 50-fold excess of biotin. The concentration of unbound spin label was estimated from the intensity of the sharp low field line (Fig. 5A, arrow 1). The dissociation of spin label II from the 4:1 complex was biphasic (Fig. 14). Approximately half of the spin labels dissociated from avidin quite rapidly (kₐ₁₀⁻⁵ s⁻¹), while the remainder dissociated at a much slower rate (kₐ₂ 1.22 × 10⁻⁶ s⁻¹). The ESR spectrum of the 4:1 complex recorded 90 min after the addition of biotin was that of a simple, noninteracting, highly immobilized spin label similar to that shown in Fig. 4G. Thus, it would appear that, when spin label II is bound to a site that is adjacent to a site occupied by another molecule of spin label, it dissociates more rapidly than when it is bound to a site that is adjacent to a site occupied by biotin. Green has estimated (1) that the dissociation rate constant for the biotin-avidin complex is 9 × 10⁻⁵ s⁻¹. No evidence was found for a biphasic dissociation of biotin from avidin. When 2 eq of spin label II were added immediately to a freshly prepared 2:1 complex of biotin and avidin, the spectrum of the bound spin label was similar to that shown in Fig. 4A. No change in the spectrum was observed after 24 hours. The same spectrum was obtained when the spin label was added to the complex 24 hours after the addition of biotin to avidin. Thus, it would appear that the distribution of biotin among the sites is random and that no redistribution of biotin occurs among the sites. Green has come to similar conclusions based on other data (13). In contrast to spin label II, the dissociation of label V from a 4:1 complex with avidin was monophasic with a dissociation rate constant of 3.85 × 10⁻⁵ s⁻¹ (Fig. 14). This suggests that when 2 molecules of label V occupy adjacent sites on avidin, there is little or no interaction between them.

**DISCUSSION**

On the basis of their studies with a series of bis-biotinyl derivatives, Green et al. have concluded that the four subunits...
of avidin are arranged with 222 symmetry and that the subunits are grouped in two pairs at each end of the short axis of the tetramer whose dimensions are 55 Å x 55 Å x 41 Å (4). The presence of dipole-dipole interactions in the ESR spectrum of the 4:1 complexes of spin labels II to IV with avidin (Fig. 5) confirms the postulate of Green et al. (4) that the biotin binding sites of avidin exist in pairs. The distance between adjacent bound nitroxides ($r_{12}$) can be calculated from the following equation.

$$r_{12} = \left( \frac{5.56 \times 10^6}{|2D|} \right)^{1/2}$$

where $|2D|$ is the maximal splitting of the peaks in gauss (5). Clearly for spin labels II to IV bound to avidin, we have a situation in which $|2D| < 2$ Å². For spin label II, the splitting is 13.8 G for both the low field and midfield doublets (Fig. 11), which gives a distance of 10 Å between adjacent nitroxide groups. In calculating this value from Equation 1, it has been assumed that the ESR spectrum of the 4:1 complex between spin label II and avidin (Fig. 5B) contains contributions from both dipolar split and noninteracting nitroxides and that contributions from other types of interaction are either nonexistent or are small enough to be neglected. Secondly, it has been assumed that it is possible to remove the contribution of the noninteracting nitroxides by spectral subtraction (Fig. 11). Thirdly, it has been assumed that the nitroxide group is a point dipole. Fourthly, it has been assumed that the molecular motion of the nitroxides is low on the ESR time scale. This would seem to be a reasonable assumption, since the ESR spectrum of the 2:1 complex between spin label II and avidin (Fig. 4G), exhibits a splitting of 69 G between high and low field extrema which is characteristic of a highly immobilized spin label. Finally, it has been assumed that the observed splitting ($|2D|$) is derived from an orientation in which the dipolar axis (i.e., the axis between the two nitroxides) is perpendicular to the z-axes of the two nitroxides. If the splitting is derived from some other orientation, then the calculated distance would be smaller.

The dipolar splittings for spin labels III and IV (Figs. 12 and 13), estimated from the midfield doublets, are 11.9 and 14.2 G, respectively. The corresponding distances between the nitroxide groups of spin labels III and IV bound to adjacent sites on avidin calculated from Equation 1 are 16.7 Å and 15.7 Å, respectively. It is of interest to note that the insertion of one piperidine ring of label II to give spin label V, no dipole-dipole interaction occurred between spin labels bound to avidin. A model for the arrangement of the binding sites in avidin which accommodates both our findings and those of Green et al. (4) is shown in Fig. 15. In this model the recognition sites for the biotin ring system are represented as clefts located at the bottom of a depression in the protein surface. The distance between the amide carbon atom and the nitroxide nitrogen atom of spin label II, in its fully extended conformation, was estimated from a Corey-Pauling-Kolton model to be about 5 Å. Thus the maximal separation between the amide carbon atoms of 2 molecules of II bound to adjacent sites on avidin is 26 Å ($2 \times 5 \text{ Å} + 16 \text{ Å}$). This value is at the upper end of the limits set by Green et al. (4) on the basis of their experiments with the biotinyl reagents. An examination of Corey-Pauling-Kolton models also reveals that the distance between the nitroxide nitrogen atom and the amide carbon atom of spin label III is about 0.5 Å less than it is in label II. It is perhaps, therefore, not surprising that the distance between the nitroxide groups of label III bound to adjacent sites is somewhat greater than that observed for label II bound to the same sites. A similar argument can also be used to explain why the nitroxide groups of avidin-bound IV are closer than those of label III. The strong immobilization of all of the avidin-bound spin labels makes it necessary to postulate the existence of accessory binding sites on the surface of avidin which can interact with the heterocyclic rings that bear the nitroxide groups (Fig. 15A). Green has proposed (10), on the basis of binding studies with HABA and other dyes, the existence of nonpolar binding sites on avidin. It seems reasonable, therefore, to suggest that the accessory sites with which the spin labels interact are hydrophobic in nature.

The ESR spectrum of the 4:1 complex of spin label II with avidin suggests that some of the bound spin labels do not undergo dipole-dipole interactions with their neighbors. The most plausible explanation for this observation is the existence of a second set of accessory hydrophobic sites on the surface of avidin which can interact with the piperidine ring of spin label II in the manner shown in Fig. 15. If adjacent nitroxide groups are separated by a distance of 30 Å, then the expected splitting of 2 G, calculated from Equation 1, would probably be too small to be detectable. The apparent increase in the proportion of spin labels undergoing dipole-dipole interactions in 0.5 N KOH (Fig. 10) may result from some pH-induced conformational change in avidin which makes interaction of the nitroxides with the adjacent accessory sites (Fig. 15A) more favorable. The lines of the low field doublet of the dipole-dipole spectrum are separated from the low field extremum of the noninteracting label by about 7 G (Fig. 5). The frequency of interconversion between these forms must therefore be significantly less than 19.6 MHz ($7 \times 2.8$ MHz) or $1.96 \times 10^7$ s⁻¹. When the 4:1 complex is heated, the frequency of interconversion between the two forms of bound spin label II shown in Fig. 15 may increase sufficiently so that time averaging occurs and the lines fuse (Fig. 9). The broadness of the lines in the ESR spectrum of the 4:1 complex at 80° (Fig. 9) suggests that there is still some interaction occurring between adjacent nitroxides. This broadening may be due to a dipole-dipole mechanism. Alternatively, the thermal motion of the avidin molecule may be sufficient to allow the nitroxides of spin labels bound to adjacent sites to come close enough together for some spin exchange to occur. Because spin exchange is brought about by direct orbital overlap, it would be necessary for the nitroxides to approach within a range of 6 Å or less. Since the ESR spectrum of avidin spin label V does not exhibit dipole-dipole interaction, it is probable that the nitroxide group of this spin label interacts solely with the second set of accessory binding sites (Fig. 15B).

The more rapid dissociation of spin label II from avidin when bound to a site adjacent to an already occupied site certainly provides a reasonable explanation for the time-dependent rearrangement of the 2:1 complex of II and avidin and the biphasic release of II from the 4:1 complex. The precise mechanism for this observation is not at present clear. The fact that neither biotin itself (13) nor spin label V (Fig. 14) undergoes such a rearrangement suggests that it is not merely the occupation of a given site by label II which is responsible for a reduction in affinity of the adjacent site for the same label. The most plausible explanation for this phenomenon is that some kind of interaction occurs between the nitroxide...
groups of spin label II bound to adjacent sites on avidin. Since the dipolar splitting suggests that these groups do not come closer than 16 Å, it would appear unlikely that steric effects are involved. There is some spectral evidence that, in solution, the canonical form $\equiv N_6\equiv O$ may make a significant contribution to the resonance structure of the nitroxide group (17). Thus it is possible that electrostatic repulsion may occur between the nitroxide groups of spin label II bound to adjacent sites on avidin.

Green has suggested that the biotin-induced blue shift in the fluorescence emission maximum of avidin was due to a decrease in the polarity of the environment of tryptophan (13). He has also suggested that the quenching of avidin tryptophan fluorescence by binding is due to some other unspecified mechanism. The molar extinction coefficient of spin label II in methanol at 336 nm is 3.96 so that it is unlikely that the quenching of avidin tryptophan fluorescence by binding is due to some other unspecified mechanism. The molar extinction coefficient of spin label II in methanol at 336 nm is 3.96 so that it is unlikely that the quenching of avidin tryptophan fluorescence by binding is due to some other unspecified mechanism. The molar extinction coefficient of spin label II in methanol at 336 nm is 3.96 so that it is unlikely that the quenching of avidin tryptophan fluorescence by binding is due to some other unspecified mechanism. The molar extinction coefficient of spin label II in methanol at 336 nm is 3.96 so that it is unlikely that the quenching of avidin tryptophan fluorescence by binding is due to some other unspecified mechanism.

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