A Spectrophotometric Assay for Avidin and Biotin Based on Binding of Dyes by Avidin

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The binding of anionic dyes by serum albumin has been studied by many workers (reviewed by Foster, 1960). The evidence suggests several cationic sites situated in hydrophobic regions of the molecule. For example, the bound dye shows spectral changes that are in accord with its location in a non-polar environment. Since it was suggested (Green, 1963b) that the biotin-binding sites of avidin were in part non-polar, the interaction of avidin with several dyes was tested by spectroscopic methods. The most striking effects were shown by 4'-hydroxyazobenzene-2-carboxylic acid, a dye that has been used for the estimation of serum albumin (Rutstein, Ingenito & Reynolds, 1953) and studied in detail by Baxter (1963, 1964). When bound by albumin the dye has a decreased extinction at 348 m\(\mu\) and a new absorption band at 480 m\(\mu\). The effects observed with avidin were even greater. Addition of the dye to an excess of avidin under conditions where almost all of it was bound gave a new absorption band at 500 m\(\mu\) (\(\varepsilon_{500}\) increased from 600 to 34500) and a change of colour from yellow to red. At the same time the 348 m\(\mu\) band of the free azo-dye anion (\(\varepsilon_{20700}\); Baxter, 1964) almost disappeared. These changes were reversed by the addition of 4 moles of biotin/mole of avidin. Similar spectral changes could be produced by diluting a neutral aqueous solution of the dye into 100 vol. of dimethylformamide.

The results of titrating the dye-binding sites at two different avidin concentrations are shown in Fig. 1 (○, △), where they are compared with curves calculated from the law of mass action. Extrapolation of the initial linear portion of the curve obtained at high concentration of avidin (○) showed that 1 dye mol. was bound/biotin-binding site (4/mol. of avidin; Green, 1964). The shape of both curves agreed fairly well with that calculated assuming a single set of sites (\(K = 5.8 \times 10^{-6} M\)) with no interactions between them. The slight departure from the calculated curve could be due either to the presence of intrinsically different types of avidin in the preparation used (D.31; Melamed & Green, 1963) or to a dependence of \(K\) on the number of sites occupied.


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The displacement of the dye by biotin is shown by the third set of points (■). The sharp end point, which was to be expected from the low dissociation constant of the avidin–biotin complex (Green, 1963a), permits an accurate titration of biotin-binding sites by using the dye as an indicator. The following procedure was found convenient. The avidin (2 ml. of 10 \(\mu\)M solution in 0.02 M-sodium phosphate or -tris–HCl buffer, pH 7.0) was mixed with dye at a concentration (100 \(\mu\)M) sufficient almost to saturate the binding sites. The decrease in extinction at 500 m\(\mu\) was then measured after successive addition of a standard solution of biotin from a pipette or micro-syringe, until no further

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**Fig. 1. Titration of avidin with 4'-hydroxyazobenzene-2-carboxylate (○, △) and of the avidin–dye complex with biotin (■).** The fraction of biotin-binding sites (\(\phi\)) occupied by dye is plotted as a function of total dye concentration (\(D\)) at two different concentrations of avidin. The concentrations of binding sites (\(A\)) were 14.1 \(\mu\)M (△) and 244 \(\mu\)M (□) in 0.02 M-sodium phosphate buffer, pH 7.0. The continuous lines were calculated from the law of mass action, assuming a single dissociation constant (\(K = 5.8 \times 10^{-6} M\)) for the avidin–dye complex, by using the relation \(2\phi = (x + \alpha + 1) - \sqrt{(x + \alpha + 1)^2 - 4x}\), where \(x = D/A\) and \(\alpha = K/A. (■)\). The fraction of biotin-binding sites occupied by dye as a function of total biotin concentration. The titrations were performed by the addition of 1 \(\mu\)l. samples of dye or biotin solution from a 10 \(\mu\)l. Hamilton micro-syringe to 0-8 or 1-6 ml. of avidin solution contained in cuvettes of 1 cm. (△) or 0.1 cm. (□) path. The concentration of bound dye was calculated from \(E_500\) by using the extinction coefficients of free and bound dye given in the text. Corrections were applied for dilution by the small volume of titrant added.
change occurred. The standardized solution of avidin was then used to estimate biotin. The avidin or biotin concentration could also be calculated from the decrease in $E_{500}$ by dividing by $\Delta E_{500}$ (34,000), provided that the component which was not being estimated was in excess. This avoids the titration procedure, but may be subject to error if other proteins are present. A change in $E_{500}$ of approx. 0.1 was produced by 50 $\mu$g. of avidin or 0.65 $\mu$g. of biotin/ml. Either version of the method is both simpler and more accurate than the alternative method based on the 233$\mu$m spectral shift of avidin (Green, 1963b).

Biotin analogues and other substances that are bound by avidin (Green, 1963b) also displace the dye, to an extent dependent on their binding constants. Only those close analogues that can compete with biotin itself affect the amount of biotin required to reach the end point. The presence of relatively weakly bound competing substances in the biotin solution may reduce the sharpness of the end point but does not affect the titre. It should be possible to use the method to titrate biotinyl residues that are covalently attached to enzymes.

The nature of the spectral change is of some interest. From the evidence of Baxter (1964), it can be said that it is probably due to the quinonoid form of the dye monoanion, which is stabilized by an intramolecular ($-\text{CO}_2\cdots\cdot\text{HN}<$) hydrogen bond when in a non-aqueous medium. This form is not favoured in water or other hydroxylic solvents that can form intermolecular bonds of comparable stability.

Baxter (1964) has also discussed the specificity of the spectral change with respect to both protein and dye components. Only serum albumins and avidin, of many proteins tested, gave new absorption bands in the 480–500$\mu$m region. The stoichiometric displacement of the dye by biotin is specific for avidin. The closely related streptavidin, kindly provided by Dr F.J. Wolf (Chaitel & Wolf, 1964), behaved in a similar manner to the avidin from egg white.

The behaviour of a number of other dyes with avidin was also investigated spectroscopically. Proflavine, alizarin, bromothymol blue, methyl orange and 4-hydroxyazobenzene showed negligible change in spectrum. 4'-Hydroxyazobenzene-4-carboxylic acid showed a new peak at 430$\mu$m ($\varepsilon$ 18 700) and methyl red (4'-dimethylaminooazobenzene-2-carboxylic acid) new peaks at 548 and 577$\mu$m. N-Phenyl-1-naphthylamine-8-sulphonic acid, which does not fluoresce in water, gave a yellow–green fluorescence ($\lambda_{\max}$ 480$\mu$m, fluorescence efficiency 0.04). All these changes were reversed by biotin. Bovine serum albumin has been shown to produce a much more marked enhancement of fluorescence of the naphthylamine derivative (Weber, & Lawrence, 1953; Weber & Young, 1964), whereas in contrast, the spectral changes induced in the other two dyes in the last group were smaller than those produced by avidin (Baxter, 1964; Klotz, Burkhard & Urquhart, 1952).

Although there are these differences between the spectral changes accompanying binding to the two proteins there is a qualitative similarity of behaviour that suggests that they have part of the binding mechanism in common. It is likely that both proteins have non-polar sites with some degree of conformational adaptability (Karush, 1950), which could account for the binding of dyes of varied structure. When biotin is bound by avidin these sites disappear, either by direct interaction with the biotin or by internal neutralization of the amino acid side chains involved.

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