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Avidin

4. STABILITY AT EXTREMES OF pH AND DISSOCIATION INTO SUB-UNITS BY GUANIDINE HYDROCHLORIDE

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The great stability of avidin over a wide pH range, particularly when combined with biotin, has frequently been noted and has been studied qualitatively by Fraenkel-Conrat, Snell & Ducay (1952*b*). In the present paper a more detailed investigation was made by the technique of dif-

ference spectrophotometry. The behaviour of avidin in concentrated solutions of urea and guanidine was also examined since it appeared likely that the molecule was built up from sub-units (Melamed & Green, 1963). The great stability, coupled with a molecular weight which was unchanged by 8*M*-urea (Fraenkel-Conrat, Snell & Ducay, 1952*a*), suggested that the sub-units might be linked covalently. Therefore attempts were made to separate

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them by reducing the disulphide bonds. However, it proved possible to bring about reversible dissociation of avidin without reduction, and this process and its relation to biotin-binding were studied by a number of techniques. A spectrophotometric study of the ionization of the tyrosine residues of avidin is also presented.

MATERIALS AND METHODS

Materials. Avidin was purified as described by Melamed & Green (1963). Guanidine hydrochloride was prepared from guanidine carbonate (AnalaR), crystallized from water and recrystallized from methanol. 1-Dimethylaminonaphthalene-5-sulphonyl chloride was a gift from Dr G. Weber.

(1-Dimethylaminonaphthalene-5-sulphonyl)avidin was prepared by the method of Weber (1952), by using about 6 moles of 1-dimethylaminonaphthalene-5-sulphonyl/mole of avidin. The reaction mixture was left overnight at 4°, the acetone was removed by dialysis for 4 hr. in the cold and the solution was run through a short column of Dowex 2 (Cl⁻ form; 200-mesh; 8% cross-linked) to remove all traces of free sulphonic acid. The biotin-binding capacity, determined by spectrophotometric titration, was unaffected by the labelling.

Denatured avidin was prepared by dissolving avidin in 6M-guanidine hydrochloride in the appropriate buffer and allowing the solution to stand overnight at room temperature.

Buffer solutions. The stated molarity of ammonium acetate buffers refers to the non-buffering species, present wholly in the ionic form. The molarity of tris buffers refers to the total concentration of tris present.

Determination of avidin. Avidin concentration was determined spectrophotometrically at 282 m μ . ($E_{1\text{cm}}^{0.1\%} = 1.57$; Melamed & Green, 1963.)

Biotin-binding activity was determined either with [¹⁴C]-biotin (Green, 1963a) or spectrophotometrically (Green, 1963b). For accurate determinations ($\pm 1\%$) a spectrophotometric titration was carried out, by using the increase in E_{233} to determine the end point. For some purposes the following rapid but less accurate spectrophotometric procedure was convenient. An excess of biotin was added to the avidin sample in a cuvette and the amount of avidin present was calculated from the increase in E_{233} ($\Delta\epsilon_{\text{mM}}$ of biotin bound = 25).

Determination of thiol groups. Thiol groups were determined by spectrophotometric titration with *p*-chloromercuribenzoate at 255 m μ in 0.1M-ammonium acetate, pH 4.6 (Boyer, 1954).

Spectrophotometry. Difference spectra were obtained with a Unicam SP. 700 recording spectrophotometer as described by Green (1963b). 6M-Guanidine hydrochloride was reasonably transparent at wavelengths above 225 m μ ($E_{225} = 0.3$), but the extinction rose rapidly at lower wavelengths ($E_{220} = 1.1$) and measurements here required cells of short light-path.

Direct measurement of spectral shift. These were kindly performed by Dr G. H. Beaven by using the logarithmic cam method of Holiday (1950) to determine the position of the tryptophan fine-structure band at about 290 m μ .

Spectrophotometric titration with acid or base. The titrations were carried out in small glass vessels or directly in 1 cm.

cuvettes with magnetic stirring, by using a Radiometer TTT1b pH-meter and G222B microelectrode (alkaline pH range). HCl (1N) or KOH (2N) was added from an Agla micrometer syringe to give the desired pH. The same volume of water was added to the reference cell, containing avidin at pH 7, and the extinction difference at the desired wavelength was determined, or occasionally the whole difference spectrum was scanned. In the acid pH region the titrations were followed at 233 m μ to detect denaturation, which is usually accompanied by exposure of the tryptophan residues (Glazer & Smith, 1961). No salt was added. In the alkaline region the titrations were carried out in 0.2M-KCl at 246 m μ , the wavelength of maximum difference for ionization of the avidin tyrosines. The avidin concentration was 0.2–0.3 mg./ml. Titrations of denatured avidin in 3M-guanidine hydrochloride were performed in the same way, with the addition of an extra pair of cuvettes containing 3M-guanidine hydrochloride for the extinction measurements. KOH was added to the solvent cuvette in the reference beam, and water to that in the sample beam, to correct for the effect of alkali on the absorption spectrum of guanidine hydrochloride. The number of tyrosine residues ionized at a given pH was calculated by using a value for $\Delta\epsilon_{\text{max}}$ of 1.0×10^4 , determined with free tyrosine (cf. Hermans, 1962).

Kinetic measurements. The rate of denaturation by guanidine hydrochloride was followed spectrophotometrically at 233 m μ , in tandem cells to correct for the extinction of guanidine hydrochloride (Green, 1963b). The measurements were mostly conducted at room temperature (22°). Since the rate went through a minimum with respect to temperature at about 20°, it was possible to obtain reproducible results at room temperature in spite of the lack of precise temperature control. In the experiment in which the denaturation in 5.3M-guanidine hydrochloride was followed in parallel with the biotin-binding, the reaction mixture (10 ml.) was incubated in a water bath at 25°. Reference solutions for the denaturation difference spectrum were prepared in separate 1 cm. cuvettes, containing avidin and guanidine hydrochloride respectively, at the appropriate concentrations. Samples (1.6 ml.) were removed from the reaction mixture at intervals, ΔE_{max} was measured or the whole spectrum was scanned, and the sample was returned to the reaction vessel. To measure the biotin-binding, duplicate 0.4 ml. samples were transferred to 1 cm. microcells, 2 μ l. of biotin solution (0.8 mg./ml.) was added to one of them, and 2 μ l. of water to the other. The value of ΔE_{233} gave the amount of avidin-biotin complex formed. Occasionally 0.2 ml. samples were transferred to one of a second pair of 1 cm. microcells and diluted with water (0.15 ml.) to give a final concentration of 3M-guanidine hydrochloride. The difference spectrum was measured against a similar sample taken at zero time. This served as a check on the interconvertibility of avidin and denatured avidin in 3M-guanidine hydrochloride.

Osmotic pressure measurement. Osmotic pressures were determined with the apparatus described by Kupke (1960) in a water bath at $25 \pm 0.01^\circ$. The height of the *n*-decane in the 1.1 mm. capillary was determined with a cathetometer relative to the height of a decane meniscus in a similar reference capillary. Equilibration took 3–6 days depending on the efficiency of the preliminary dialysis. It was considered complete when the overnight pressure change was less than 0.1 mm.

Measurements with the ultracentrifuge. These were performed by Dr R. H. Pain. Sedimentation constants were determined with a Spinco model E analytical ultracentrifuge. Sedimentation was continued for 8 hr. at 59 780 rev./min. and 19°. The diffusion coefficients were calculated by the height-area method from the photograph at 444 min. The partial specific volume was taken as 0.72 (0.73 less 1% to allow for the 6M-guanidine hydrochloride in the solvent; Kielley & Harrington, 1960). Molecular weight was also determined from a sedimentation equilibrium experiment, by using 'method III' of Van Holde & Baldwin (1958). A solution of denatured avidin in a solution of 6M-guanidine hydrochloride in 0.1M-tris-HCl buffer, pH 8.0, was diluted with an equal volume of the same buffer and dialysed for 72 hr. against a solution of 3M-guanidine hydrochloride in 0.1M-tris-HCl buffer, pH 8.0. A 1.3 mm. column of this solution was employed for the sedimentation experiment.

Fluorescence polarization. The polarization of fluorescence of (1-dimethylaminonaphthalene-5-sulphonyl)avidin (0.15 mg./ml.) was determined at temperatures between 2° and 40°, by using the photoelectric polarization photometer of Weber (1956) with polarized exciting light. The exciting light was filtered through 2 cm. of 2% (w/v) copper sulphate and two Corning 5970 filters. The fluorescence was observed through a pair of Corning filters (3486 and 4303). Mean rotational relaxation times, ρ_h , were calculated from the relation (Weber, 1952):

$$1 + \frac{3\tau_0}{\rho_h} = \frac{1/p - 1/3}{1/p_0 - 1/3}$$

where p is the observed polarization, p_0 the polarization in a viscous medium [60% (w/v) sucrose at 2°], and τ_0 , the lifetime of the excited state of the 1-dimethylaminonaphthalene-5-sulphonyl label, was assumed to be 13 μ sec. (Steiner & McAlister, 1957). In addition, p_0 was determined by extrapolation of the graph of $1/p$ against T/η . The experimental value of ρ_h was compared with ρ_0 ($= 3\eta M^2/RT$), the rotational relaxation time of an anhydrous spherical molecule of the same molecular weight. Values of ρ_h/ρ_0 greater than 1 are due to asymmetry or hydration or both; values less than 1 indicate translational or rotational dissociation or both (Steiner & Edelhoch, 1961). The viscosity of 3M-guanidine hydrochloride solutions was determined with an Ostwald viscometer at 5° intervals from 2° to 40°, to obtain the required values of T/η .

Optical rotation. Values of $[\alpha]_D$ were determined with a Hilger Microptic photoelectric polarimeter. The protein concentration was about 1%. Values of $[\alpha]_D$ were reproducible to $\pm 0.5^\circ$.

Experiments with reduced avidin. For these experiments solutions were prepared in water that had been deoxygenated by boiling. They were gassed with O_2 -free N_2 immediately before use and care was taken to exclude air at all stages where the pH of the mixture was above 6. Reduction was first attempted in urea. Avidin (10 mg.) was dissolved in 1 ml. of a solution of urea (9M) in 50 mM-ammonium acetate, pH 9. Mercaptoethanol (5 μ l.) was added. After 2 hr. at room temperature the protein was precipitated with a mixture of acetone and N-HCl (39:1, v/v) and freed from urea and mercaptoethanol by repeated washing with acetone-N-HCl and acetone (White, 1960). Reduction of avidin (10 mg./ml.) was also carried out in 6M-guanidine hydrochloride in 50 mM-ammonium acetate, pH 9, for 4 hr.

at room temperature. The reaction mixture was then treated, according to the experiment to be performed, as follows:

(1) For biotin-binding activity, determination of thiol groups and reoxidation. The reduction mixture (1 ml.) was run through a column (1 cm. $\times$ 20 cm.) of Sephadex G-25 in 0.1N-acetic acid (Anfinsen & Haber, 1961). The protein-containing fractions were collected in N_2 -filled tubes and the pooled fractions were kept under N_2 . Samples, to which a small amount of mercaptoethanol (1 mM) was added, were taken for activity measurements. Other samples were taken for determinations of thiol groups which were performed without delay.

(2) For sedimentation or optical rotation in 6M-guanidine hydrochloride. The reduced avidin (16 mg./ml.) was mixed with an equal volume of 6M-guanidine hydrochloride in 50 mM-ammonium acetate, pH 4.3 (final pH 4.5). The solution was dialysed with rocking for 62 hr. against 6M-guanidine hydrochloride containing the same concentration of ammonium acetate buffer and mercaptoethanol. A control sample of unreduced avidin was treated in the same way, omitting mercaptoethanol throughout. The solution was centrifuged at 15 000 rev./min. for 30 min. in the SW39 head of a Spinco model L ultracentrifuge to remove any aggregated material. No precipitate was visible at the bottom of the tube after centrifuging. The sedimentation constant or optical rotation was determined as described above.

(3) For osmotic pressure measurements in 3M-guanidine hydrochloride. The thiol groups of the reduced avidin and the excess of mercaptoethanol were carboxymethylated by the addition of 1M-triethylamine iodoacetate, the pH being maintained at 9.0 by the simultaneous addition of 1M-triethylamine. When the pH ceased to fall and the nitroprusside reaction was negative, the solution was acidified to pH 6 with acetic acid and then dialysed overnight against 10 mM-acetic acid at 4°. Then 0.1 vol. of 1M-tris-HCl buffer, pH 8.0, was added together with sufficient solid guanidine hydrochloride to give a concentration of 3.0M. This avidin solution (2.9 mg./ml.) was then dialysed for 72 hr. against 3M-guanidine hydrochloride in 0.1M-tris-HCl, pH 8.0, and the osmotic pressure was measured.

Reoxidation of reduced avidin. Reduced avidin from the Sephadex column (1.3 mg./ml.) was diluted with an equal volume of 0.2M-tris-HCl buffer, pH 7.3, and sufficient 2M-tris was added to neutralize the free acetic acid. The solution was allowed to stand as a thin (5 mm.) layer in air for 3 days at 4° to reoxidize the thiol groups. Precipitated protein was spun down and the supernatant was assayed with [^{14}C]-biotin.

RESULTS

Reduction of disulphide bonds of avidin. When avidin was reduced in the presence of 9M-urea, its biotin-binding activity was unchanged and no thiol groups could be detected by spectrophotometric titration with *p*-chloromercuribenzoate. Since 9M-urea produces a red shift with avidin (Green, 1963b) rather than a denaturation blue shift, it was perhaps not surprising that the disulphide bonds were not reduced under these conditions. Other denaturing agents were therefore examined and it was found that either sodium dodecylsulphonate

(0.1M) or guanidine hydrochloride (6M) brought about a blue shift of the absorption spectrum, indicating increased exposure of the tryptophan to the aqueous environment. The resulting difference spectrum in 6M-guanidine hydrochloride is shown in Fig. 1. To a first approximation it may be regarded as a mirror image of the biotin-induced difference spectrum (Green, 1963*b*) reflected in the plane of the base-line, as would be expected if it were simply due to a blue shift of the tryptophan absorption bands. The short wavelength peak was shifted slightly, to 231 m μ , and was somewhat broader than the corresponding peak due to biotin. In the long-wavelength region the main difference was the absence of the shoulder at 300 m μ . The denaturation by 6M-guanidine hydrochloride was accompanied by loss of biotin-binding activity (no spectral change on adding biotin) but that this could be immediately and completely restored by diluting the guanidine hydrochloride to 0.6M. In contrast, the spectrum of the avidin-biotin complex was not affected by 6M-guanidine hydrochloride, even at 60°, thus confirming the great stability of this complex. More detailed kinetic and equilibrium studies on this system are described below. The denatured avidin was reduced with mercaptoethanol, and, after 1.5 hr. at 22°, less than 1% of the original activity could be detected, by the [¹⁴C]-biotin method, in a sample that had been diluted 1:30 in 3mN-hydrochloric acid. This result was confirmed in the following experiment in which parallel samples of avidin were denatured in 6M-guanidine hydrochloride at pH 9.3. One sample was reduced (0.5 mg. of mercaptoethanol/mg. of avidin) and duplicate 1:20 dilutions were made in 1 cm. cuvettes. Biotin was added to one of the cuvettes and the difference spectrum was run. It

showed no significant departure from the base-line, whereas a parallel experiment in the absence of mercaptoethanol gave a normal difference spectrum ($\Delta E_{233}/E_{282} = 0.88$).

Attempts to measure the extent of reduction by spectrophotometric titration with *p*-chloromercuribenzoate, after the removal of guanidine hydrochloride and mercaptoethanol on Sephadex in 0.1N-acetic acid, were unsuccessful. The reduced avidin solution remained clear when the pH was raised to 4.6, but the addition of *p*-chloromercuribenzoate led to turbidity that increased on standing. It was therefore impossible to determine the end point with any certainty. Attempts to restore the biotin-binding activity by slow reoxidation at pH 7.3 of the fractions from the Sephadex column were also unsuccessful. There was some precipitation of insoluble protein, but neither the soluble nor the insoluble product bound any [¹⁴C]biotin.

It may be concluded from these experiments that avidin is reversibly inactivated by 6M-guanidine hydrochloride but not by 9M-urea. The disulphide bonds could only be reduced when the avidin was inactivated and reduction rendered the inactivation irreversible. The avidin-biotin complex was not denatured by 6M-guanidine hydrochloride.

Molecular weight of avidin in guanidine hydrochloride solution. Previous measurements of the molecular weight of avidin by physical methods (Fraenkel-Conrat *et al.* 1952*a*) gave values between 55 000 and 70 000. Melamed & Green (1963) concluded, from measurements of biotin-binding capacity, that the correct value is about 53 000. To determine whether the avidin sub-units are linked by disulphide bonds the sedimentation behaviour of avidin in 6M-guanidine hydrochloride was compared with that of an identical solution to which mercaptoethanol had been added. Both samples gave single symmetrical boundaries and low sedimentation constants. The results in Table 1 show that even without reduction the molecule was dissociated into sub-units. The apparent slight increase of molecular weight on reduction was probably a consequence of increased asymmetry of the reduced avidin.

Sedimentation behaviour in strong solutions of guanidine hydrochloride is often difficult to interpret owing to interactions between denatured molecules and consequent strong dependence of the sedimentation constant on concentration (Kielley & Harrington, 1960). It was therefore not possible from this experiment to deduce the number of sub-units formed/molecule. Further measurements of molecular weight were made by sedimentation-equilibrium (Van Holde & Baldwin, 1958) and osmotic-pressure methods (Kupke, 1960). These were carried out in 3M-guanidine hydrochloride rather than in 6M-guanidine hydrochloride to

Fig. 1. Difference spectrum of avidin in 6M-guanidine hydrochloride minus native avidin. The experiments were carried out in 50 mM-sodium phosphate buffer, pH 6.8. The right-hand ordinate scale for points above 255 m μ has been expanded by a factor of 2.5. Points below 229 m μ were obtained in a separate experiment in 1 mm. cuvettes, to eliminate stray-light errors due to high extinction of guanidine in this region.

decrease the density, viscosity and salt concentration. It is shown below that avidin that has been denatured in 6M-guanidine hydrochloride remains so in 3M-guanidine hydrochloride. There was good agreement between the values obtained by the different methods and, although no great accuracy can be claimed, since concentration-dependence was not studied, it seems clear that in 6M-guanidine hydrochloride avidin dissociates into sub-units of about the same size as the biotin-binding unit. This conclusion received some support from measurements of fluorescence polarization described below. Reduction of the disulphide bonds did not affect the molecular weight.

Reversibility of the denaturation of avidin by guanidine hydrochloride. The extent and reversi-

bility of the denaturation was studied as a function of concentration of guanidine hydrochloride. The parameter ΔE_{231} was used as a measure of denaturation. At low concentration of guanidine hydrochloride there was a slight red shift, and the blue shift did not commence until the concentration of guanidine hydrochloride reached 3-4M. It was maximal at 5.5M and then decreased again. This decrease was a non-specific red shift due to the almost complete exposure of the tryptophan residues in the denatured avidin to guanidine hydrochloride solutions of increasing refractive index (Bigelow & Geschwind, 1960; and Table 2 below). It was much greater than that shown by native avidin in 1-3M-guanidine hydrochloride where the tryptophan residues are partially buried. The same

Table 1. *Molecular weight of denatured avidin, before and after reduction*

In all experiments the avidin was denatured in 6M-guanidine hydrochloride, as described in the Materials and Methods section. This solution was diluted with an equal volume of buffer for the experiments in 3M-guanidine hydrochloride.

Method	Concn. of protein (mg./ml.)	Concn. of guanidine hydrochloride in medium (M)	Reduction	$S_{20,w}$ (s)	$10^7 \times D_{20,w}$ (cm. ² sec. ⁻¹)	f/f_0	Mol. wt.
Sedimentation velocity	8	6	.	0.89	4.5	3.7	16 400
	8	6	+	0.76	3.2	4.8	19 500
Sedimentation equilibrium	3	3	.	.	.	.	18 700
Osmotic pressure	8.9	3	.	.	.	.	17 000
	2.6	3	.	.	.	.	20 000
	1.7	3	.	.	.	.	16 000
	2.9	3	+	.	.	.	16 000

Table 2. *Positions of the fine-structure peak (at about 291 m μ) of avidin and of N-acetyltryptophan methyl ester*

Expt. 1 gives the wavelength in native avidin and avidin-biotin complex. Expt. 2 shows the position after denaturation and reversal. Expt. 3 is a repeat of Expt. 2 in the presence of mercaptoethanol. Expt. 4 was a control experiment with α -N-acetyltryptophan methyl ester. For Expts. 2 and 3 a stock solution of denatured avidin (4 mg./ml.) in a solution of 6M-guanidine hydrochloride in 0.12M-ammonium acetate, pH 9, was divided in two, and mercaptoethanol (2 mg./ml.) was added to one half. Then 0.15 ml. samples of each were diluted to 2.4 ml. with (a) 6M-guanidine hydrochloride made 56 mN with respect to acetic acid and (b) 56 mN-acetic acid. After determination of the position of the fine-structure peak, 1 equiv. of biotin was added to (b) and the position was redetermined (c). The final pH was 4.6 in all cases. The values of $\Delta E_{\max}/E_{282}$ and of $[\alpha_D]$ were determined in separate experiments.

Expt. no.	Concn. of guanidine hydrochloride (M)	Biotin	λ (Å) (± 1 Å)	$\Delta\lambda$ (Å)	$\Delta E_{\max}/E_{282}$	$[\alpha_D] \pm 0.5^\circ$
Avidin 1	0	+	2916	5	0.94	-22
	6	+	2917	6	1.24	.
	0	.	2911	0	0	-23
	6	.	2901	-10	-0.65	-64
Avidin 2 (a)	0.37	.	2912	1	.	.
(b)	0.37	+	2920	9	0.90	.
Avidin 3 (a)	6	.	2905	-6	.	-76
(b)	0.37	.	2913	2	.	.
(c)	0.37	+	2913	2	.	.
α -N-Acetyltryptophan methyl ester	0	.	2884	0	0	.
	3	.	2890	6	0.66	.
	6	.	2896	12	1.5	.

effect was responsible for the increased blue shift that was found when denatured avidin solutions were diluted. This increase continued until the guanidine hydrochloride concentration was below 3M, when reversal of the denaturation commenced. The marked hysteresis showed that it was possible to keep native or denatured avidin in 3M-guanidine hydrochloride without appreciable interconversion, thus allowing a direct comparison of their properties in the same solvent system. Even over periods of several days no spectral changes were observed when native or denatured avidin, or mixtures of the two, were allowed to stand in 3M-guanidine hydrochloride. This absence of interconversion was also found with partially denatured systems, where samples were removed during a kinetic run and diluted to give a guanidine hydrochloride concentration of 3M. The effect of the addition of biotin to the avidin-guanidine hydrochloride system under various conditions is shown in Fig. 2 (filled points). Both the spectrophotometric titration and the difference extinction coefficients were unaffected by concentrations of guanidine hydrochloride below 3M. After denaturation commenced the magnitude of the red shift caused by the addition of biotin decreased in proportion to the extent of denaturation. The combination with biotin remained instantaneous and no further increase in the red shift occurred over periods of 1-2 days. Similarly, the addition of biotin to the completely denatured avidin had no effect over long periods.

Biotin-induced reassociation only became appreciable when the concentration of guanidine hydrochloride fell to 3M. The addition of 1 equiv. of biotin then led to a slow red shift ($t_{\frac{1}{2}} = 4$ hr.), although in the absence of biotin the avidin showed no signs of reassociation over periods of 6-7 days. As the guanidine hydrochloride was further diluted the rate of the change increased (at 2.5M, $t_{\frac{1}{2}} = 40$ min.; at 2.1M, $t_{\frac{1}{2}} = 20$ sec.) until at 1.7M it was too fast to measure. Spectrophotometric titration of the reassociated avidin showed between 90 and 95% of the original biotin-binding capacity was retained and the value of $\Delta E_{233}/E_{282}$ was 0.90 ± 0.05 .

Kinetics of denaturation of avidin by guanidine hydrochloride. The rate of denaturation was followed spectrophotometrically at concentrations of guanidine hydrochloride between 4.5 and 6.5M. The reaction resembled the urea denaturation of ovalbumin (Simpson & Kauzmann, 1953) in that it was not a simple first-order process but could be approximated by the sum of several exponential terms. A detailed analysis was not attempted, but a measure of the initial rate was obtained by plotting the data by the method of Guggenheim (1926), by using short time-intervals (Fig. 3). The resulting rate constants, expressed as half-times, were

strongly dependent on concentration of guanidine hydrochloride and a plot of $\log t_{\frac{1}{2}}$ against $\log [\text{guanidine hydrochloride}]$ gave a straight line of slope 12 (Fig. 4). This can be interpreted as an apparent reaction order of 12 with respect to guanidine hydrochloride (Simpson & Kauzmann, 1953). When avidin, renatured by dilution of the

Fig. 2. Denaturation blue shift as a function of concentration of guanidine hydrochloride. The experiments were carried out in 50 mM-sodium phosphate buffer, pH 6.8. O, Forward reaction: identical avidin samples were allowed to stand overnight in increasing concentrations of guanidine hydrochloride; a parallel series of tubes contained solvent of the same concentration of guanidine hydrochloride for use in one of the tandem reference cells; the other reference cell contained avidin. ●, Effect of adding 1 equiv. of biotin after denaturation. Δ, Reverse reaction: avidin, denatured in 6.6M-guanidine hydrochloride overnight, was diluted 1:20 into the appropriate concentration of guanidine hydrochloride; ΔE_{231} was measured after 18 hr. ▲, Effect of adding 1 equiv. of biotin after the dilution.

Fig. 3. Rate of denaturation of avidin in a solution of 6.4M-guanidine hydrochloride in 50 mM-sodium phosphate buffer, pH 6.8, at 22°. A Guggenheim plot was used with E_{231} as a measure of denaturation. $\log(E_t - E_{(t+10)})$ was plotted against t (min.).

guanidine hydrochloride, was denatured a second time (in 6M-guanidine hydrochloride) the rate was the same as that found with the original sample (Fig. 4).

Simpson & Kauzmann (1953) presented evidence that the complex kinetics of ovalbumin denaturation were due to a sequence of first-order changes leading to a progressive increase in laevorotation. If a similar mechanism were operative here one might expect to find that loss of biotin-binding activity was associated with one of these steps and therefore that it should follow simple first-order kinetics. Biotin-binding was therefore measured in parallel with the denaturation blue shift in 5.3M-guanidine hydrochloride. The results (Fig. 5) showed no systematic departure from inverse proportionality between the red shift induced by biotin and the denaturation blue shift. This suggests that denaturation as measured by spectral shift is a one-step process (probably dissociation into sub-units) accompanied by loss of activity, and that the complex kinetics may be due to the presence of several avidin species of different stability. The chromatographic data on the avidin used in these experiments (Melamed & Green, 1963) reveal the presence of more than one active component, which is consistent with this hypothesis.

A single experiment on denaturation of the pervaporated avidin (Melamed & Green, 1963), in which 40% of the tryptophan was oxidized, showed that much lower concentrations of guanidine hydrochloride would bring about denaturation. The rate was appreciable in 2M-guanidine hydrochloride and the reaction went to completion in 4M-guanidine hydrochloride in a few hours.

Direct measurement of spectral shifts. Since $\Delta E_{\max}/E_{282}$ is proportional to $\Delta\epsilon$ it is also proportional to $\Delta\lambda$ provided that one is considering a simple spectral shift without marked intensity changes. Provided also that the relative shifts of the 220 m μ and 280 m μ aromatic absorption bands do not change, the relative values of $\Delta E_{\max}/E_{282}$ should be comparable with the shift of the 290 m μ fine-structure peak measured directly by the logarithmic cam method. There was qualitative agreement between the two sets of results (Table 2). The quantitative differences could have been due in part to experimental error, but it is also likely that the basic assumptions are only approximations. The logarithmic cam method showed a red shift of 5 Å when avidin and biotin combined and a blue shift of 10 Å when avidin was denatured. Reduction of the disulphide bonds surprisingly decreased the denaturation blue shift to 6 Å. Restoration of the normal avidin spectrum on dilution of the 6M-guanidine hydrochloride (to 0.38M) was confirmed, and the addition of biotin to this produced the usual red shift (Table 2: Expt. 2). Dilution of the reduced

avidin also restored the fine-structure band to its normal position, but in this case the addition of biotin had no effect (Expt. 3). The experiment with *N*-acetyltryptophan methyl ester showed that guanidine hydrochloride produced a red shift, approximately proportional to its concentration, of 2 Å/mole of guanidine hydrochloride/l. This is the non-specific red shift referred to above, which is a function of the refractive index of the medium. It was the same, within $\pm 15\%$, for a given refractive index, whether the solvent was glycol (Green, 1963b) or guanidine hydrochloride. The shift measured in terms of $\Delta E_{\max}/E_{282}$ was not linear

Fig. 4. Denaturation kinetics: effect of concentration of guanidine hydrochloride on the half-time for the initial phase of the reaction. The experiments were carried out in 50 mM-sodium phosphate buffer, pH 6.8. O, Native avidin; Δ, renatured avidin.

Fig. 5. Relation between biotin-induced red shift and denaturation blue shift during a kinetic run in 5.3M-guanidine hydrochloride. The line is the expected relation for a transition involving simultaneous exposure of tryptophan residues and loss of activity.

with the concentration of guanidine hydrochloride. The upward curvature observed (Table 2; and unpublished values) was similar to that found with *N*-acetyltryptophan in glycol (Green, 1963*b*, Fig. 3).

Optical rotation of avidin. Measurements of optical rotation are also included in Table 2. Denaturation caused a considerable increase in laevorotation but combination with biotin had no significant effect. Even after reduction of the disulphide bonds the rotation was still far from the value for a completely unfolded (random-coil) structure (-110° ; Yang & Doty, 1957). The optical rotation of avidin in 9*M*-urea (-33°) indicated some unfolding, whereas that of the avidin-biotin complex (-24°) was not affected by urea.

Behaviour of avidin at extremes of pH. The rate of denaturation of avidin increased as the pH was lowered. This effect was not studied in detail, but some preliminary measurements of a blue shift accompanying denaturation at acid pH in the absence of guanidine hydrochloride are given in Fig. 6. Denaturation commenced below pH 2. The final magnitude of the blue shift at pH 1 depended in a peculiar way on the time for which the avidin was allowed to stand at pH values below 2 before it was finally adjusted to pH 1. The shorter the time at intermediate pH the greater the final blue shift. If the pH was lowered directly to 1, then a maximum value of $\Delta E_{233}/E_{282}$ of -0.55 was reached. When the titration was reversed the protein did not begin to regain its native configuration until the pH had risen to 3. Biotin-binding activity was restored at the same time.

The study of the ionization of tyrosine at alkaline pH was complicated by the presence of large amounts of tryptophan. Fortunately, the alkali-induced difference maxima of the two amino acids in the short-wavelength region occur at different wavelengths (tryptophan 233 $m\mu$; tyrosine 246 $m\mu$) and it proved possible to distinguish tyrosine ionization from biotin-induced red shifts or denaturation blue shifts of the tryptophan spectrum. Spectrophotometric titration of the avidin tyrosines at 246 $m\mu$ (Fig. 7) showed that at least three out of the four tyrosine residues/molecule ionized with a *pK* of about 12.3. The exact number was difficult to determine as it was impossible to reach a sufficiently high pH to complete the titration. The curve was less steep than the theoretical one for ionization of groups with a single intrinsic *pK* and there was probably a slight spread in the *pK* values. The final difference spectrum was typical of tyrosine (Hermans, 1962; Paiva & Paiva, 1962) with only slight effects due to tryptophan at 233 and 294 $m\mu$, suggesting that little denaturation had occurred. This was confirmed by the reverse-titration curve which followed the forward curve within 0.2 pH

unit, and by spectrophotometric titration with biotin after neutralization, which showed only 5–10% inactivation, depending on the time for which the avidin had been left at high pH. The apparent shift of about 0.2 pH unit in the reverse-titration curve may have been due to an upward displacement of the curve resulting from slight turbidity and precipitation that occurred during titration to alkaline pH. Titration of denatured avidin in 3*M*-guanidine hydrochloride showed normal ionization of 3.9 tyrosine residues/molecule with a *pK* of 10.1. The avidin-biotin complex showed only 20% ionization at pH 13. This remarkable stability of avidin at high pH was convincingly confirmed by a spectrophotometric titration with biotin in 0.5*N*-potassium hydroxide by using tandem reference

Fig. 6. Denaturation blue shift as a function of pH. HCl (1*N*) was added from a micrometer syringe to one of a pair of similar cuvettes containing avidin (0.3 mg./ml.). The extinction difference at 233 $m\mu$ was recorded after time had been allowed for a steady reading to be obtained. ○, Forward titration; the final solution (pH 1) was left overnight before commencing the reverse titration (Δ). ●, Extinction difference obtained when pH was lowered directly from 7 to 1.

Fig. 7. Ionization of tyrosine residues in avidin and the avidin-biotin complex. The titration was with 2*N*-KOH. Δ, Forward titration of avidin in 0.2*M*-KCl; ▲, reverse titration (with 2*N*-HCl); □, forward titration of avidin-biotin complex; ○, forward titration of avidin in 3*M*-guanidine hydrochloride; ●, reverse titration in 3*M*-guanidine hydrochloride. The curve joining the circles is the theoretical for the ionization of 3.9 tyrosine residues of *pK* 10.05.

cells containing 0.5N-potassium hydroxide and avidin at neutral pH (Fig. 8). The addition of biotin under these conditions led to simultaneous red shift of the tryptophan spectrum (followed at 233 $m\mu$) and blue shift of the tyrosine spectrum (followed at 246 $m\mu$) due to uptake of a proton accompanying the binding of each biotin molecule. The same end point was obtained at both wavelengths.

Fluorescence polarization of native and denatured avidin and of the avidin-biotin complex. The polarization of the fluorescence of avidin labelled with 1-dimethylaminonaphthalene-5-sulphonyl groups was followed as a function of T/η under two sets of conditions (Fig. 9). In the first experiment avidin was compared with the avidin-biotin complex in water at pH 6.8, and in the second avidin was compared with denatured avidin, both in 3M-guanidine hydrochloride at pH 6.8. The extrapolated values of p_0 for avidin (4.20) and the avidin-biotin complex (4.35) agreed well with the values in 60% (w/v) sucrose. For native and denatured avidin in 3M-guanidine hydrochloride extrapolated values were considerably higher than the values in sucrose. It is possible that the difference is due to the linear extrapolation. A slight curvature of the plot of $1/p$ against T/η would suffice to bring the two sets of values into line. A similar explanation was considered by Steiner & Edelhoch (1961) for the high extrapolated values of p_0 that they observed with thyroglobulin denatured by urea or sodium dodecylsulphonate. Relaxation times were only computed for the first experiment (avidin and avidin-biotin complex). Uncertainty

about the value of τ_0 in 3M-guanidine hydrochloride (Steiner & Edelhoch, 1961) and in the extrapolated value of p_0 did not permit a meaningful estimate of ρ_h under these conditions, and interpretation was confined to a comparison of the slopes of the linear portions of the T/η plots. The values of ρ_h and ρ_0 (calculated for anhydrous spheres of mol.wt. 53 000) for avidin and the avidin-biotin complex at 25° are given in Table 3. The value of ρ_h/ρ_0 for avidin was much closer to 1 than has yet been observed with other proteins [e.g. ovalbumin, bovine serum albumin (Weber, 1952) and lysozyme (Steiner & McAlister, 1957)] where ρ_h/ρ_0 is about 2. This could be due either to the greater symmetry and compactness of avidin or to some freedom of internal rotation of the sub-units with respect to each other (Weber, 1952). If the second explanation were correct one would expect that the avidin-biotin complex, which is more stable than avidin and presumably possesses a

Fig. 9. Fluorescence polarization of (1-dimethylaminonaphthalene-5-sulphonyl)avidin. $1/p$ was measured as a function of T in 50 mM-sodium phosphate buffer, pH 6.8. The points at $T/\eta = 0$ were obtained by the addition of sucrose (2 g./ml.) and cooling to 2°. ○, (1-Dimethylaminonaphthalene-5-sulphonyl)avidin; □, (1-dimethylaminonaphthalene-5-sulphonyl)avidin-biotin complex; ●, (1-dimethylaminonaphthalene-5-sulphonyl)avidin in 3M-guanidine hydrochloride; △, renatured (1-dimethylaminonaphthalene-5-sulphonyl)avidin in 3M-guanidine hydrochloride; ▲, denatured (1-dimethylaminonaphthalene-5-sulphonyl)avidin in 3M-guanidine hydrochloride.

Fig. 8. Spectrophotometric titration of avidin with biotin in 0.5N-KOH. ●, Red shift of tryptophan at 233 $m\mu$; ■, blue shift of tyrosine at 246 $m\mu$. The titration was completed in 10 min. to minimize inactivation at the high pH.

Table 3. Fluorescence polarization of (1-dimethylaminonaphthalene-5-sulphonyl)avidin and (1-dimethylaminonaphthalene-5-sulphonyl)avidin-biotin complex

The experiments were carried out in 50 mM-sodium phosphate buffer, pH 6.8, at 25°. $\rho_0 = 3\eta M\bar{v}/RT$, where $M = 53\ 000$ and $\bar{v} = 0.73$ ml./g. of DNS (1-dimethylaminonaphthalene-5-sulphonyl).

	$1/\rho_0$	ρ_h ($m\mu/\text{sec.}$)	ρ_0 ($m\mu/\text{sec.}$)	ρ_h/ρ_0
DNS-avidin	2.64	56	42	1.34
DNS-avidin-biotin complex	2.74	50	42	1.19

more rigid structure, would have a larger value of ρ_h . The fact that ρ_h is smaller supports the first hypothesis. The curvature of the plot of $1/p$ against T/η at higher temperatures suggests onset of internal oscillations of these sub-units, which would appear to be greater in avidin than in the avidin-biotin complex.

The slopes in 3M-guanidine hydrochloride should be proportional to the molecular volumes of native and denatured avidin (Weber, 1952), provided that there is no change in molecular shape. Since the asymmetry almost certainly increases on denaturation the observed ratio of slopes of 1.9 indicates dissociation of the molecule into more than two sub-units. The only evidence so far presented that the avidin-biotin complex does not dissociate in 6M-guanidine hydrochloride is that there was no denaturation blue shift in this solvent, and that a change in molecular weight of avidin, when measured, has been accompanied by such a shift. The converse does not necessarily hold, so to check against possible dissociation of the avidin-biotin complex its fluorescence polarization in 6M-guanidine hydrochloride was compared at 25° with that of avidin in the same solvent. When (1-dimethylaminonaphthalene-5-sulphonyl)avidin was mixed with guanidine hydrochloride the initial value of $1/p$ was 3.9 and it increased slowly to 6.5. The corresponding values for the avidin-biotin complex were 4.26 and, after 18 hr., 4.34. The low constant value of $1/p$ shows that dissociation was insignificant. The difference (0.36) from the initial reading with avidin (3.9) was similar to that between the two proteins in water.

DISCUSSION

Denaturation difference spectrum. The difference spectrum (Fig. 1) is approximately the inverse of that due to biotin. In particular, the relative magnitudes of the peaks in the 230 m μ and 290 m μ regions are similar. This suggests that most of the extinction difference in the 230 m μ region could be due to a shift of the tryptophan spectrum (Green, 1963b), but in view of the increased laevorotation (Table 2) some contribution from changes in peptide absorption are also likely. Glazer & Smith (1961) have obtained similar difference spectra accompanying the denaturation of several proteins and have placed particular emphasis on the latter contribution. It is likely that the relative importance of the two factors differs from one protein to another and from one set of conditions to another. It may be possible to assess this when more information on the relative magnitude of the shifts of the short- and long-wavelength aromatic absorption bonds is available. It should then be possible to make an estimate of the aromatic contribution at

230 m μ from the observed shifts at 290 m μ , where other factors do not interfere. It is possible to make an approximate estimate of the extent of exposure of the tryptophan residues in denatured avidin from a comparison of the position of the fine-structure peak with that of *N*-acetyltryptophan methyl ester in the same solvent (6M-guanidine hydrochloride). There was only 5 Å difference between the two positions (Table 2), which is less than 20 % of the difference between *N*-acetyltryptophan methyl ester and avidin in water, suggesting almost complete exposure of the tryptophan residues in denatured avidin. Reduction of the disulphide bonds did not increase this further, nor did it greatly affect the optical rotation, showing that even in 6M-guanidine hydrochloride, in the absence of stabilizing disulphide linkages, avidin still possesses an appreciable amount of secondary and tertiary structure.

Stability of avidin. The stability of avidin, particularly when combined with biotin, under a wide variety of conditions has been commented on by most previous workers (e.g. Fraenkel-Conrat *et al.* 1952a, b) and is amply confirmed here. The absence of a blue shift and the resistance to reduction by mercaptoethanol in 9M-urea is unusual. It can be correlated with the high concentration of guanidine hydrochloride (about 4M) required to effect the same change, since it has been shown that, mole for mole, guanidine hydrochloride is about three times as effective a denaturing agent as urea (Schellman, Simpson & Kauzmann, 1953). Urea also has a special relationship to avidin, since it appears to be bound at the biotin-binding site (Green, 1963b) and this may offset its denaturing properties. The increase of laevorotation from -23° to -34° shows that 9M-urea does produce some disorganization of the molecule, though not sufficient to affect the biotin-binding.

Perhaps the most remarkable feature of the stability is the behaviour at alkaline pH. The reversible ionization of protein-bound tyrosine at pH 12.3 is unprecedented and the uptake of a proton after the addition of biotin under these conditions is even more surprising. The increased pK of tyrosine in many proteins has often been ascribed to the formation of tyrosine-carboxylate hydrogen bonds (Scheraga, 1961). If such a link exists in avidin one would expect to find three or four carboxyl groups/molecule with a pK lowered by about 2 pH units. It may be suggested speculatively that the blue shift of the tryptophan spectrum below pH 2 is due to the uptake of protons by such groups. Its magnitude, compared with the small effect on tryptophan at pH 13, might be due to the net positive charge on the molecule, which would be much larger than the net negative charge at pH 13. It is not yet known whether this bonding

of the phenolic hydroxyl group of tyrosine is essential for the binding of biotin or how much it contributes to the stability of the avidin-biotin complex. It seems impossible to check this by ionizing the tyrosines since their pK in the avidin-biotin complex is too high, so that some specific method for irreversibly blocking them would be necessary. Fraenkel-Conrat *et al.* (1952*b*) have reported little effect of iodination of avidin on the biotin-binding activity, but it is possible that under the conditions used the tyrosine residues were buried and were not iodinated.

The blue shift observed at acid pH is probably correlated with the decreased affinity of avidin for various analogues at low pH (Green, 1963*b*) and with the increased rate of exchange of biotin into the radioactive avidin-biotin complex at pH 2 (N. M. Green, unpublished work). The blue shift was smaller than that observed with guanidine hydrochloride and no measurements of molecular weight have been made to check the possibility of dissociation.

Reversible dissociation of avidin into sub-units. At present there is no direct evidence to show how far the blue shift and loss in activity are correlated with changes in molecular weight. The limited molecular-weight data show that avidin is dissociated into sub-units by 6M-guanidine hydrochloride and remains so when the guanidine hydrochloride is diluted to 3M, but there is no evidence concerning the molecular weight at intermediate degrees of denaturation. The fluorescence-polarization measurements showed (a) that native avidin in 3M-guanidine hydrochloride was not dissociated, since the slope of the plot of $1/p$ against T/η was unchanged by 3M-guanidine hydrochloride and (b) that the avidin-biotin complex did not dissociate in 6M-guanidine hydrochloride. It seems most likely that the denaturation is an 'all-or-none' process involving simultaneous dissociation into sub-units, exposure of the tryptophan residues, loss of activity and partial loss of helical structure. It is possible that some of the helical structure was lost before dissociation although not sufficient to prevent biotin-binding. The almost complete reversal of denaturation which followed the removal of the guanidine hydrochloride is further evidence of the great stability of the native conformation of avidin. Even after storage for weeks at 4°, or months at -15°, solutions of avidin in 6M-guanidine hydrochloride regained 90-95% of their original activity when the guanidine hydrochloride was diluted. Further evidence for the regain of the native structure is provided by fluorescence-polarization (Fig. 9) and denaturation-rate measurements (Fig. 4) on the renatured protein. The rapid and complete reassociation of the sub-units after dilution of the guanidine suggested that their conformation was

not greatly disturbed in 6M-guanidine hydrochloride. This lack of disturbance is probably due to the disulphide bonds, since reduction of these led to irreversible inactivation.

The reversal of protein denaturation as a function of concentration of urea or guanidine hydrochloride has not often been studied, and the only well-established example of hysteresis between the forward and reverse branches of the curve appears to be the denaturation of γ -globulin by urea (Edelhoch, Lippoldt & Steiner, 1962). It is possible that the hysteresis is due to the unmasking of additional guanidine-binding sites as a consequence of denaturation. There are difficulties that prevent an exact interpretation of present data so that the following discussion can only be regarded as tentative. When the avidin dissociates the additional binding sites would become saturated with guanidine hydrochloride and reassociation would be prevented until the concentration had been lowered sufficiently for the guanidine hydrochloride to dissociate once more. From Fig. 2 this would appear to occur at about 2.5-3M-guanidine hydrochloride. A difficulty arises when the effect of addition of biotin to this system is considered. A slow formation of the avidin-biotin complex was observed, in which the rate-limiting step was probably the formation of some dimeric or trimeric form of avidin which then combined rapidly with the biotin. Now, once the biotin is bound the great stability of the avidin-biotin complex would prevent any reversal of the association of the sub-units so that renaturation should go to completion. However, in 3M-guanidine hydrochloride, for example, it did not do so in 18 hr. It is possible to explain this with assumptions about heterogeneity of avidin similar to those used to explain the complex kinetics of the denaturation reaction. If several avidin species were present which reassociated at different rates, dependent on a high power of the concentration of guanidine hydrochloride, then 18 hr. might well be insufficient time for all these reactions to go to completion. At slightly higher concentrations of guanidine hydrochloride (3.5M) the addition of biotin brought about no change in 18 hr., showing that there were no species present that could interact with the biotin to form a stable complex. This implies that guanidine was still bound to the unmasked sites and that reassociation was prevented. Therefore, provided that denaturation can begin in 3.5M-guanidine hydrochloride, it should eventually go to completion. Fig. 2 shows that, after 18 hr., denaturation was only partial, but this may again be a question of rates and of heterogeneity of the avidin. These difficulties can only be resolved by further experiments with a homogeneous avidin preparation and with more attention to the rate of attainment of equilibrium.

The question of the relation of the binding sites to the sub-units remains unsettled. The great stability of the avidin-biotin complex in concentrated solutions of guanidine hydrochloride shows that, directly or indirectly, biotin assists in the cohesion of the sub-units. The exposure of the tryptophan residues on dissociation and the greater ease of dissociation when the tryptophan residues are partially oxidized suggest that they play some part in holding the sub-units together in addition to their role in biotin-binding. These observations are directly explicable if the biotin-binding sites are situated between adjoining surfaces of the sub-units, and this hypothesis is favoured on the grounds of simplicity. However, if each binding site were in the interior of the sub-unit, rather than on its surface, it would still be possible to explain these results as secondary consequences of the biotin-binding, due to accompanying conformational changes of the avidin.

SUMMARY

1. Reduction of the disulphide bonds of avidin was dependent on prior denaturation by guanidine hydrochloride.

2. Molecular weights from sedimentation-diffusion, sedimentation, equilibrium and osmotic-pressure measurements showed that even without reduction avidin dissociated into three sub-units in 6M-guanidine hydrochloride.

3. Dissociation in guanidine hydrochloride solution was accompanied by a denaturation blue shift of the tryptophan absorption bands and by loss of biotin-binding activity. It could be reversed with regain of the normal spectrum and activity by diluting the guanidine hydrochloride tenfold. The reversal showed marked hysteresis with a concentration difference of 2.8M-guanidine hydrochloride between the forward and reverse branches of the curve.

4. Throughout the whole course of the denaturation the loss of biotin-binding activity was proportional to the denaturation blue shift. This is most readily explicable in terms of a single-step reaction involving simultaneous loss of activity, exposure of tryptophan residues to the solvent and dissociation into sub-units.

5. Avidin was also reversibly denatured below pH 2. Again, the reversal, as measured by the blue shift, was accompanied by hysteresis with a pH difference of 1.5 units between the forward and reverse branches of the curve.

6. No loss of biotin-binding activity was observed on standing for short times at alkaline pH, and spectrophotometric titration with biotin could be performed in 0.5M-potassium hydroxide. The tyrosine residues, which in avidin ionized reversibly

at pH 12.3, regained a proton in 0.5N-potassium hydroxide when biotin was added. The tyrosine residues of denatured avidin in 3M-guanidine hydrochloride showed normal ionization (pK 10.1).

7. Measurement of the polarization of fluorescence of avidin labelled with 1-dimethylaminonaphthalene-5-sulphonyl groups was consistent with a compact symmetrical conformation of avidin, which changed little when biotin was added. Denaturation approximately halved the rotational relaxation time, which was consistent with the results of molecular weight measurement.

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