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Heat Stability of Avidin and Avidin-Biotin Complex and Influence of Ionic Strength on Affinity of Avidin for Biotin.* (29576)

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Avidin, a toxic protein, which renders biotin unavailable for animal and microbial growth combines firmly with biotin to yield an avidin-biotin complex with a dissociation constant of 10^{-15} M(1). Biotin, when combined with avidin, cannot be liberated from the avidin-biotin complex by ordinary proteolytic enzymes(2), and the complex is stable over a wide pH range(3,4). It has been reported, however, that bound biotin is completely released from the complex by steam sterilization(3). The microbiological assay (5) of avidin is based on this property. In a study of a new method for determination of avidin by use of radioactive biotin and gel-filtration, conducted by the present authors (6), it was discovered that the so-called heat labile complex is quite stable in 0.2 M ammonium carbonate solution. For example, it has been found that only a very small portion of biotin is dissociated from the complex in a solution of 0.2 M ammonium carbonate at 100°C for 15 minutes. Similar results were obtained by the use of other pH buffer solutions of the same ionic strength. In addition, in solutions of low ionic strength the affinity of avidin for biotin is much less than in solutions of high ionic strength. The present paper reports the results from these experiments on the relationship of heat and ionic strength to the formation and dissociation of the avidin-biotin complex. Similar conclusions with respect to the stability of the avidin-biotin complex to heat and the protective effect of ions on the stability of the complex recently have been reported by Pai and Lichstein(7).

Materials and methods. D-Biotin-carboxyl-C^{14} with a specific activity of 22.9 mc/millimole was kindly provided by Dr. O. Wiss, Hoffmann-La Roche, Basel, Switzerland.

Avidin was purchased from Nutritional Biochemicals Co., Cleveland, Ohio, with an indicated activity of 2.5 units/mg. Pure avidin also was prepared by a new method of Melamed and Green (M. & G.)(8) with an activity of 13.1 units/mg.

The experimental procedures involved in separation of avidin-biotin from biotin by gel-filtration chromatography on a Sephadex G-25 column have been described(6). The dimensions of the column used were 0.8 cm × 33 cm.

The heat stability experiments involved both a study of avidin stability prior to com-

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bination with biotin as well as a study of avidin stability subsequent to combination. In the experiments involving the heat stability of uncombined avidin, 1 ml aliquots of avidin solution in buffer containing about 0.4 units of avidin were mixed with 4 ml amounts of buffer in 10 ml volumetric flasks and the mixtures heated for definite times at definite temperatures. In the experiments involving the heat stability of the avidin-biotin complex, 1 ml aliquots of avidin solution in buffer containing about 0.4 units of avidin were mixed with 4 ml amounts of biotin-carboxyl-C^{14}, containing 1 μg of biotin, in 10 ml volumetric flasks and the mixtures heated for definite times at definite temperatures. Following heat treatment of either series, the flasks were removed from the heat source and immediately frozen in a dry ice-acetone mixture and kept in a freezer. The frozen solutions were warmed to room temperature in running warm water (about 1 min) just prior to chromatography. In the case of the heated uncombined avidin, 4 ml of biotin, containing 1 μg of biotin carboxyl-C^{14}, were added to the flasks and the contents diluted to 10 ml with buffer. In the case of the heated avidin-biotin complex the contents of the flasks were diluted to 10 ml with buffer. In all cases Sephadex chromatography was completed within 24 hours of the time the initial heat treatments were begun. One ml aliquots of the various solutions were used for chromatography. Fractions of 6 drops (0.575 ml) were collected directly in 20 ml scintillation counting vials placed on an automatic fraction collector with a drop counting unit. Ten ml of Bray's solution(9) were added to each vial, and the vials were counted by means of a Packard Tricarb Scintillation spectrometer operating at an efficiency of 36%.

The experiments involving the effect of ionic strength on formation of the avidin-biotin complex and release of biotin from combination involved the mixing of avidin solutions and biotin solutions of various ionic strengths followed by Sephadex chromatography. In each instance the column was developed with the same buffer that was used as the medium for the reaction between avidin and biotin. Specifically, about 0.4 units of avidin in 1 ml of appropriate buffer and 1 μg of D-biotin-carboxyl-C^{14} in the same buffer were mixed in a 10 ml volumetric flask. The flask was shaken gently for 15 minutes and then diluted to 10 ml with the buffer under study. An aliquot of 1 ml of this reaction mixture was applied to the Sephadex column. The column was then eluted with the buffer. Appropriate fractions were collected and counted in the liquid scintillation spectrometer as described. From the radioactivity appearing in the first chromatographic peaks (avidin-biotin) the extent of the reaction between avidin and biotin was calculated.

In a variation of the experiments involving the effect of ionic strength on formation of the avidin-biotin complex, avidin and excess biotin-carboxyl-C^{14} in ammonium carbonate solution of various ionic strengths were mixed and dialyzed against a large volume of the solution used in making the original solutions. At 24-hour intervals the dialyzed solutions were examined for radioactivity as a measure of the effect of ionic strength on the stability of the avidin-biotin complex to dialysis.

Results. Results of the heat stability studies involving 0.2 M ammonium carbonate as the solvent and steaming at 100°C are summarized in Fig. 1. It is apparent that uncombined avidin is very labile to steaming. On the other hand, the avidin-biotin combination is unexpectedly stable to heat treatment. This statement is true irrespective of the avidin source. For example, at the end of 15 minutes of steaming at 100°C only about 10% of the biotin was dissociated from the complex when N.B.C. avidin was used and none was dissociated from the complex when M. & G. avidin was used. At the end of steaming for 2 hours 35% of avidin-biotin complex remained in the experiment with N.B.C. avidin and 45% of avidin-biotin complex remained in the experiment with M. & G. avidin. Under more drastic conditions, 120°C for 15 minutes in the autoclave, the avidin-biotin complex is completely dissociated (Fig. 2). The stability of the avidin-

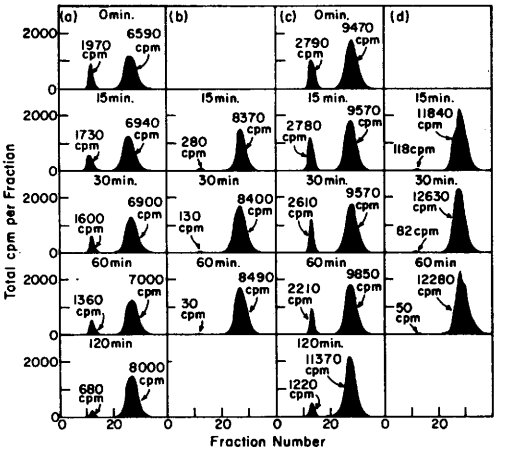

FIG. 1. Summary of heat stability studies. (a) and (b): Avidin used was purchased from N.B.C.; (c) and (d): Avidin used was prepared by the method of M. & G. (a) and (c): Biotin was added to the avidin before heat treatment. These data represent the heat stability of the avidin-biotin complex. (b) and (d): Biotin was added to avidin after heat treatment. These data represent the heat stability of uncombined avidin. The first peak in each chromatogram represents the avidin-biotin complex. The second peak in each chromatogram represents excess unreacted biotin.

biotin combination is not a function of ammonium carbonate since essentially the same stability was found in tris, succinate, or acetate buffer of the same ionic strength (0.6 μ) (Table I).

As demonstrated by the data of Fig. 3, the affinity of avidin for biotin is a direct function of ionic strength. With avidin and biotin dissolved in distilled water essentially no combination occurs. With increasing concentrations of buffer a maximum level of combination occurs. There are differences in the shape of the affinity *vs* concentration curves with the various buffers that are not presently explainable. Combination of avidin with biotin would appear to take place best in ammonium carbonate solution of ionic strength greater than about 0.6 μ.

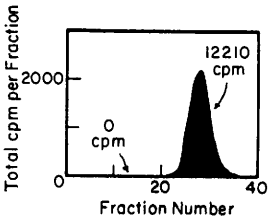

FIG. 2. Effect of autoclaving at 120°C for 15 min on the avidin-biotin complex.

TABLE I. Heat Stability of the Avidin-Biotin Complex in Buffer Solutions at 100°C.

Medium	Time of heating, min	Avidin-biotin complex remaining, %
Tris-HCl, $\mu = .6$	15	70
pH 9	60	56
Succinate, $\mu = .6$	15	80
pH 6	60	71
Acetate, $\mu = .6$	15	90
pH 5	60	80

μ = Ionic strength.

When avidin and excess biotin in ammonium carbonate where $\mu = 0.6$ are dialyzed against ammonium carbonate, $\mu = 0.6$, the avidin-biotin combination is stable (Table II). On the other hand, when avidin and excess biotin in ammonium carbonate where $\mu = 0.05$ are dialyzed against ammonium carbonate, $\mu = 0.05$, the combination is not nearly so stable. Seventy % of the biotin remaining in combination with avidin in ammonium carbonate where $\mu = 0.6$ could be dialyzed away from the avidin-biotin com-

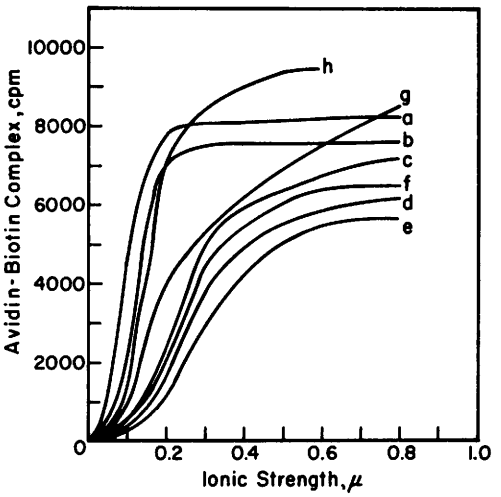

FIG. 3. Effect of ionic strength on formation of the avidin-biotin complex. (a) Ammonia-ammonium chloride buffer, pH 10; (b) ammonia-ammonium chloride buffer, pH 9; (c) Tris-HCl buffer, pH 8; (d) Tris-HCl buffer, pH 7; (e) acetic acid-acetate buffer, pH 5.8; (f) acetic acid-acetate buffer, pH 5; (g) acetic acid-acetate buffer, pH 4; (h) ammonium carbonate, pH 8.9.

TABLE II. Affinity of Avidin for Biotin as Influenced by Ionic Strength. Dialysis experiments.

Composition	Time of dialysis, hr 0	24	48
	Undialyzed material, cpm		
Biotin in ammonium carbonate, $\mu = .6$	5560	20	20
Biotin + avidin in ammonium carbonate, $\mu = .6$	4720	2240	2290
Biotin in ammonium carbonate, $\mu = .05$	5790	20	20
Biotin + avidin in ammonium carbonate, $\mu = .05$	5600	620	700

μ = Ionic strength.

bination when the ammonium carbonate concentration was $\mu = 0.05$.

Discussion. These studies demonstrate that: (a) uncombined avidin is relatively heat labile, (b) the avidin-biotin complex is relatively heat stable, (c) avidin has essentially no affinity for biotin in aqueous solution, and (d) the affinity of avidin for biotin is a direct function of ionic strength of the medium.

It would appear that avidin in aqueous solution alone probably exists in a random coil configuration. As such, avidin has no affinity for biotin. With increase in ionic strength the molecule assumes a configuration that attracts biotin. On combining with biotin the molecule must undergo a second configurational change that is responsible for the marked increase in heat stability over the heat stability of the uncombined avidin.

Although the present experiments do not afford sufficient data for calculation of equilibrium coefficients it is apparent from the dialysis experiments that the reaction between avidin and biotin is reversible when the ionic strength of the medium is reduced. Whether or not the influence of ionic strength on the affinity of avidin for biotin represents a mechanism for regulating biotin-catalyzed reactions remains to be determined.

The avidin-biotin system represents an interesting system for study of the effect of environment on the configuration of a protein. The system is especially attractive now that avidin may be readily obtained in the pure state(8) and that avidin may be readily determined by a rapid and accurate method (6).

Summary. An assay method involving the use of radioactive biotin and Sephadex chromatography was used to determine avidin and/or the avidin-biotin complex. It was demonstrated that avidin is relatively heat labile while the avidin-biotin complex is relatively heat stable. It was found that the affinity of avidin for biotin, essentially zero in aqueous solution, is a direct function of the ionic strength of the medium in which avidin and biotin react.

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