SINGLE-STEP PURIFICATION OF AVIDIN FROM EGG WHITE BY AFFINITY CHROMATOGRAPHY ON BIOCYTIN-SEPHAROSE COLUMNS

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Specific and rapid purification of enzymes can be achieved by chromatography on columns containing a highly porous, crosslinked dextran, agarose, to which an inhibitory substrate analogue has been covalently attached (Cuatrecasas, et al., 1968). The principles and techniques of affinity chromatography, previously illustrated with staphylococcal nuclease, α-chymotrypsin, and carboxypeptidase A (Cuatrecasas, et al., 1968), should be generally useful in the isolation of many proteins which are capable of binding, specifically, small molecules. Haptene-Sepharose columns, prepared by these techniques, have now been used to purify anti-DNP (Goetzle, et al., 1968) and antisulfanilic acid (Kallos, 1968) antibodies from serum. The present report describes the striking results obtained on application of affinity chromatography to the purification of avidin, a protein abundant in egg white and in the genital tracts of animals.

Highly purified avidin has been obtained from egg white by procedures which use gradient elution from three successive carboxymethylcellulose columns (Melamed and Green, 1963). McCormick (1965) achieved some degree of purification of 10% pure avidin by using columns of cellulose to which biotin had been esterified. Such columns, however, are relatively ineffective because their affinity for avidin is weak, as evidenced by the incomplete

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adsorption of the avidin applied, the ease of avidin elution by merely lowering the ionic strength of the buffer, and the incomplete separation of the avidin peak from the peak of extraneous proteins (McCormick, 1965). The shortcomings of such cellulose preparations as affinity adsorbents are especially noteworthy in view of the extremely small dissociation constant, 10^{-15} M (Green, 1963a), of the avidin-biotin complex.

In the present studies an avidin-specific adsorbent was prepared by coupling biocytin (ε-N-biotinyl-L-lysine) to Sepharose. The lysyl substitution on the carboxyl group does not impair binding to avidin (Wright, et al., 1950; Green, 1963b), and the amino group of this derivative can be conviently coupled to Sepharose. In this affinity adsorbent the structural units of biotin that are critical for avidin binding, the ureido and thiophan rings, are very distant from the solid matrix. This spatial separation minimizes steric interference with the reversible binding process; this is an important factor in affinity chromatography, as demonstrated previously for chymotrypsin-specific adsorbents (Cuatrecasas, et al., 1968).

Materials and Methods

Sepharose 4B was obtained from Pharmacia, cyanogen bromide from Eastman, D-biotin from Calbiochem, D-biotin (carbonyl ^{14}C), specific activity 233 μc/mg, from Nuclear Chicago, avidin (AF 8CA) from Worthington, and dry egg white (67B-0720) from Sigma. Biocytin (L511, 199-0-2) was a generous gift from Dr. Max Tishler, Merck Sharp & Dohme Research Laboratories.

Biocytin was coupled to cyanogen bromide-activated Sepharose as described previously (Cuatrecasas, et al., 1968). About 25 mg of biocytin were used to prepare 20 ml of substituted Sepharose. Avidin levels were estimated on the basis of binding capacities for biotin ^{14}C, using equilibrium dialysis or chromatography on Sephadex G-25 (fine grade). The purity of avidin preparations was checked by the specific activity of biotin binding (Melamed and Green, 1963), as well as by spectrophotometric titrations with biotin (Green, 1963b).

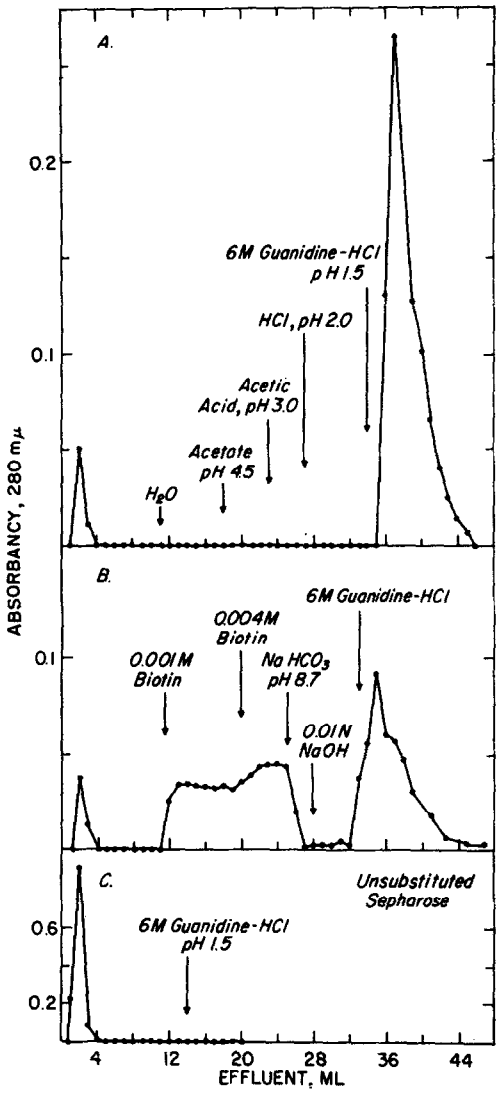

Figure 1 - Affinity chromatography of purified avidin on biocytin-Sepharose (A,B) and unsubstituted Sepharose (C) columns. The columns (0.5 x 5 cm) were equilibrated with 0.2 M $NaHCO_3$, pH 8.7, and 0.75 mg of avidin (in 0.5 ml of the same buffer) was applied to each column. One ml fractions were collected, the flow rate was about 30 ml per hour, and the experiments were performed at room temperature. Elution was attempted by varying the conditions as indicated (arrows). The small protein peak that emerges early in A and B represents an impurity which does not bind biotin ^{14}C.

Results and Discussion

Avidin is adsorbed very strongly to biocytin-Sepharose columns (Figure 1, A and B), in contrast to its total lack of affinity to unsubstituted Sepharose (Figure 1, C). Binding of avidin to these columns was so strong that elution

did not occur with extremes of pH (1.5 to 12), or with 3 M guanidine-HCl (Figure 1). Addition of biotin to the buffer in concentrations that approach the limits of solubility results in relatively slow elution of the protein (Figure 1, B). With 6 M guanidine-HCl, pH 5.2, the protein is removed slowly and comes off the column quite dilute. The most effective and useful way to elute avidin is to combine two conditions, 6 M guanidine-HCl and a pH of 1.5, that separately cause reversible denaturation of native avidin but which have no effect on the avidin-biotin complex in vitro (Green, 1963c).

The avidin eluted from the biocytin-Sepharose columns by denaturation with 6 M guanidine-HCl, pH 1.5, is completely and rapidly reconstituted by ten-fold dilution with buffer, or by dialysis to lower the concentration of guanidine. Such material can then be completely adsorbed on a biocytin-Sepharose column, has optimal capacity for binding biotin ^{14}C, and undergoes maximal spectral changes at 233 mμ on titration with biotin.

Recovery of avidin added to biocytin-Sepharose columns was about 90%. Although such columns could be used effectively several times, the capacity for avidin decreased after passing large volumes of strong acid through the column.

Hydrolysis of the biocytin-Sepharose with 6N HCl (21 hrs), followed by amino acid analysis, revealed that about one μmole of lysine (or biocytin) was present per ml of Sepharose. The theoretical capacity of such an adsorbent for avidin is therefore about 50 mg of protein per ml of Sepharose.

The biocytin-Sepharose adsorbent can be used to obtain a 4000-fold purification of avidin from crude egg white, in a single step.
About 90% of the avidin activity of egg white was readily extracted. The avidin obtained in these experiments was quite pure, as judged: a) by its capacity to bind 14.6 μg of biotin ^{14}C per mg of protein, compared to the values of 14.0 μg obtained with the pure avidin standard, and 13.8 μg described by Melamed and Green (1963); and b) by the criterium of spectrophotometric titration with biotin, which gave a value of $\Delta E_{233}/E_{282}$ of 1.10,

Table I

Purification of Avidin From Egg White by Biocytin-Sepharose Chromatography

Sample	Protein (Mg)	Avidin (Mg)	Specific Activity+	Purification
Egg white	8000	2.2	0.0037	----
Washings of Sepharose	8000	0.2	<0.0003	----
Elution from Sepharose	1.5*	1.6	14.6	4000

+ μg of biotin ^{14}C/mg of protein.

* Using $E_{1\ cm}^{0.1\%}$ of 1.57 (Melamed and Green, 1963).

Two ml of biocytin-Sepharose were added to a solution (25 ml) of 0.2 M $NaHCO_3$, pH 8.7, containing 8 gm of crude, dried egg white. After a few minutes the solution was centrifuged at 20,000 x g for 10 minutes, and the Sepharose was suspended in bicarbonate buffer and poured on a 1 x 5 cm column. The column was washed with about 25 ml of 3 M guanidine-HCl, pH 4.5, and the avidin was then eluted with 6 M guanidine-HCl, pH 1.5. Avidin concentration was determined by addition of biotin ^{14}C, followed by dialysis for 24 hours at room temperature against large volumes of 0.1 M $NaHCO_3$, pH 8.2; it was assumed that 13.8 μg of biotin are bound per mg of avidin (Melamed and Green, 1963).

compared to 0.96 for the avidin standard, and 0.94 reported by Green (1963b). When procedures similar to those described in Table I were applied to the raw egg white of a single egg, approximately 1.1 mg of avidin were obtained.

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