available commercially. A spectrophotometric method of greater accuracy but lower sensitivity, based on a biotin-induced spectral shift (Green, 1962), is presented by Green (1963a).

SUMMARY

1. [2'-¹⁴C]Biotin has been synthesized from ¹⁴CO in 70 % radiochemical yield.

2. A rapid and simple assay for avidin has been worked out, based on the determination of the excess of [¹⁴C]biotin after adsorption of the avidin-biotin complex on carboxymethylcellulose. The method can also be used for the determination of unlabelled biotin.

3. Preliminary kinetic measurements show that the rate constant for avidin-biotin combination ($7 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$) is of the same order as that for a number of enzyme-substrate-combination reactions.

4. The rate constant for dissociation was very low ($9 \times 10^{-8} \text{ sec}^{-1}$), which accounts for the apparent irreversibility of the avidin inhibition of biotin-containing enzymes. The dissociation constant of the complex calculated from the rate constants was about 10^{-15} M , corresponding to a free energy change of 20 kcal./mole of biotin bound.

I thank Professor A. Neuberger, F.R.S., who first suggested to me that a further investigation of avidin would be of interest. He also read the manuscript and made many valuable suggestions. This work was supported by a grant from the Nuffield Foundation.

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Biochem. J. (1963) **89**, 591

Avidin

2. PURIFICATION AND COMPOSITION

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(Received 19 March 1963)

Avidin preparations of high activity were obtained by Fraenkel-Conrat, Snell & Ducau (1952a). They purified this material by adsorption on bentonite, followed by selective elution with phosphate buffer and fractionation with ammonium sulphate. The isolation was complicated by the formation of slightly soluble complexes between the

basic avidin and two acidic components of egg white, a glycoprotein and a low-molecular-weight DNA. The free avidin was a basic protein of molecular weight about 60 000 and isoelectric point 10.5. It contained 10 % of carbohydrate and an unusually large amount (6 %) of tryptophan. The most active preparations bound 1 mole of biotin/30 000 g. It was notable for its stability, particularly when combined with biotin, over a wide range of pH and towards a variety of proteo-

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lytic enzymes, but it was peculiarly sensitive to light especially in the presence of Fe^{2+} and some other metal ions.

In the present work the purification has been carried out with carboxymethylcellulose and no trouble arose from complex-formation. Presumably the carboxymethylcellulose, being an acidic polymer, competes effectively for the avidin with any acidic macromolecules present. Carboxymethylcellulose was employed in the fractionation of egg proteins by Rhodes, Azari & Feeney (1958), who isolated the major components by stepwise elution of the adsorbed proteins with buffers of increasing pH. Avidin appeared as a small peak together with some unidentified globulins, but it was not obtained in quantity.

MATERIALS AND METHODS

Materials

Carboxymethylcellulose. Whatman CM70 carboxymethylcellulose (500 g.) was washed several times by decantation to remove fines and then packed into a column (100 cm. $\times$ 9 cm.) and washed in sequence with 0.5 N-NaOH containing NaCl (0.5 M) (3 l.), distilled water (50 l.), 1 mM-EDTA (25 l.) and deionized water (50 l.). After the washing the column was unpacked and the carboxymethylcellulose was sucked dry on a Buchner funnel without any paper and initially with very gentle suction. This proved to be a useful method for filtration of the rather fibrous Whatman carboxymethylcellulose, provided that it was present as a thick suspension. The prolonged washing with water was necessary to remove small amounts of soluble carboxymethylcellulose which formed soluble complexes with the avidin (Green, 1963*a*). The object of washing with EDTA was to remove metal ions which are known to contribute to the instability of avidin (Fraenkel-Conrat *et al.* 1952*a*). For the same reason deionized water and AnalaR grade ammonium acetate, ammonium carbonate and ammonia were used throughout the purification. The final carboxymethylcellulose filter-cake contained 83% of water. It was stored at 4°.

Amberlite CG-50 (type II). Fines were removed by decantation and the resin was cycled twice between the Na^+ and H^+ forms.

Buffers. Buffers of the appropriate ionic strength were prepared as follows: Ammonium acetate (1 M) was adjusted to the desired pH with ammonia and diluted appropriately. The ionic strength is equal to the molar concentration of acetate ion. Borate buffers were prepared from borax and sodium hydroxide solutions of the same Na^+ ion concentration, which was equal to the desired ionic strength.

Other chemicals. Ethylene glycol monomethyl ether was redistilled after the addition of stannous chloride (5 g./l.). Ninhydrin was of analytical grade.

Chromatography of proteins

Carboxymethylcellulose columns. The washed carboxymethylcellulose was suspended in the appropriate buffer, degassed, poured into columns and packed down as the liquid flowed through, first under gravity and finally under

an air pressure of a few pounds/in.². The resulting columns had high flow rates that sometimes had to be decreased by the use of capillary-column exits. For adsorption of proteins from dilute solution flow rates of 0.2–0.5 cm./min. were satisfactory, but for elution slower running (0.1–0.2 cm./min.) was desirable. The final loading of the columns was less than 2 mg. of protein/ml. of column volume where good resolution was desired, but for concentration of a protein solution up to 5 mg./ml. could be applied. The proteins were eluted by using linear concentration gradients of ammonium acetate or ammonium carbonate. When it was necessary to lower the pH of columns equilibrated with ammonium carbonate to below 8 they were first washed with ammonium acetate to prevent the formation of CO_2 bubbles in the columns, and similarly ammonium acetate at pH 9 was used to raise the pH of columns above 8. Fractions equivalent to 1–2 cm. of column height were collected with a drop-counting fraction collector.

Amberlite CG-50 columns. In general the method followed was that of Hirs, Moore & Stein (1953). Columns were packed with the Na^+ form of the resin which was washed with 1 mM-EDTA and then with the buffer in which the column was to be run (2 cm./hr.) until the pH of the effluent reached that of the inflowing buffer. The proteins were applied to the column as a concentrated solution (1–5%, w/v) in a buffer at the pH at which the column was to be run but at a much lower ionic strength (0.2–0.1). The proteins were eluted either with a single buffer of the appropriate ionic strength or with a shallow linear concentration gradient. Flow rates of 2 cm./hr. and loadings of 1 mg. of protein/2.5 ml. of column volume were employed.

Concentration of fractions from the columns. After determination of protein concentration and biotin-binding activity, the appropriate fractions were pooled, acidified to pH 6 with acetic acid, degassed and concentrated by adsorption on short columns of carboxymethylcellulose (Sober, Gutter, Wyckoff & Peterson, 1956). These columns were washed with 50 mM-ammonium acetate, pH 9, and the protein was eluted with 2% (w/v) ammonium carbonate. The concentrated protein solution was dialysed on a rocking dialyser against deionized water and freeze-dried.

Assay procedures. The extinction at 280 μm was used as a measure of protein concentration. Biotin-binding activity was usually determined with [¹⁴C]biotin (Green, 1963*a*). The final specific activity of the purified avidin was measured by a more accurate spectrophotometric procedure (Green, 1963*b*).

Specific activities were initially expressed as $\mu\text{g.}$ of biotin bound/ml. of protein solution of $E_{280} = 1.0$. Subsequently these values were converted into $\mu\text{g.}$ of biotin/mg. of protein by using the value of $E_{1\text{cm}}^{0.1\%}$ determined with pure avidin (1.57), and these units are used throughout the present paper.

Analytical methods

Dry weights. The protein in a Pregl 'weighing pig' was dried to constant weight in a vacuum drying 'pistol' over P_2O_5 at 110°. The heating was started 15–20 min. before the evacuation. After 1 hr. at 110° and at a pressure of 0.01 mm. Hg, the 'pistol' was closed, removed and allowed to cool. Dry air was let in and the protein plus 'pig' were weighed on a microbalance. The same procedure was followed for taring the 'weighing pig'.

Total nitrogen. Samples of solution containing approx. 0.05 mg. of nitrogen were weighed out in thick-walled tubes

(outside diameter 8 mm., wall thickness 2 mm. and length about 8 cm.). The solutions were dried *in vacuo* and 0.05 ml. of digestion mixture was added. Fresh digestion mixture was made up each time from sulphuric acid (microanalytical reagent grade). Anhydrous copper sulphate (0.2%) and selenium (0.04%) were added, and the mixture was brought to the boil and allowed to cool. The tubes were sealed, rotated to mix the contents and heated in a muffle furnace at 480° for 30 min. (Jacobs, 1960, 1962).

After being cooled, the tubes were scratched and opened with a hot glass rod. The contents were transferred, with thorough rinsing by a Pasteur pipette, to a 5 ml. volumetric flask. Then 0.5 ml. of 2M-sodium citrate buffer, pH 5, was added and the contents were titrated to pH 5 with 50% (w/w) NaOH by checking samples (0.5 µl.) externally on indicator paper. The flask was then made to the mark and replicate 1 ml. samples were transferred to test tubes fitted with rubber stoppers. Then 1 ml. of ninhydrin reagent (Moore & Stein, 1954) was added and the stoppered tubes were heated at 100° for 15 min. The tubes were cooled and 8 ml. of aq. 50% (v/v) ethanol was added to each. The extinction was measured at 563 mµ. Blanks were carried through in an identical manner with distilled water replacing the sample solution for digestion.

Total phosphorus. This was determined by the Fiske & Subbarow method as modified by Bartlett (1959).

Sugars. Hexose was determined by the orcinol-sulphuric acid procedure of Winzler (1955) as modified by François, Marshall & Neuberger (1962), with mannose as standard. Hexosamine was determined by the method of Kraan & Muir (1957) after hydrolysis in 4N-HCl at 100° (Johansen, Marshall & Neuberger, 1960; Marshall & Neuberger, 1960).

Amino acids. Analyses were performed on a Beckman Spinco model 120 amino acid analyser according to the method of Moore & Stein as described by Crumpton & Wilkinson (1963). Cystine was determined in a separate run as cysteic acid after performic acid oxidation (Moore, 1963).

Biotin content of avidin preparation. This was determined by denaturing a solution of avidin at 110° in a sealed tube for 60 min., and then allowing the released biotin to form a complex with a known quantity of native avidin. The residual activity of the native avidin was back-titrated with [¹⁴C]biotin (Green, 1963a).

Avidin assay in single eggs (cf. Green, 1963a). The egg white was homogenized with an equal volume of distilled water. One-fifth of this solution was stirred with 1 g. of carboxymethylcellulose filter-cake, and the pH was adjusted to 7 with 2N-acetic acid. The suspension was centrifuged briefly to bring down the carboxymethylcellulose with adsorbed basic proteins and the supernatant was discarded. The carboxymethylcellulose was washed twice by resuspension in 10 ml. of 20 mM-ammonium acetate, centrifuged and finally suspended in 50 ml. of distilled water. To 1 ml. of this suspension was added 0.3 ml. of [¹⁴C]biotin solution containing 3000 counts/min. in 0.1M-pyridine-acetate buffer, pH 5. The suspension was shaken for a few minutes and then the cellulose was centrifuged down. The supernatant was plated and the excess of [¹⁴C]-biotin counted. The biotin removed was taken to be equivalent to the avidin adsorbed on the cellulose.

Amide nitrogen. Avidin (5 mg.) was hydrolysed in 2N-HCl (1 ml.) at 100° in a sealed tube for 3 hr. Ammonia and basic peptides were precipitated by the addition of 1 ml. of 30% (w/v) phosphotungstic acid (Johansen *et al.* 1960) and

the mixture was left in the cold overnight. The precipitate was spun down, washed with 1 ml. of 30% phosphotungstic acid and finally made up in 10 ml. of 0.2M-sodium acetate buffer, pH 6. This procedure gives a slightly turbid solution which should be free of the hexosamine liberated by hydrolysis. Samples were incubated in Conway microdiffusion units overnight at 5° with an equal volume of saturated K₂CO₃ (Tracey, 1952). After the incubation the contents of the centre well (0.2 ml. of sodium citrate buffer, pH 5) were transferred to a test tube, together with the washings (0.8 ml.). Moore & Stein (1954) ninhydrin reagent (1 ml.) was added and the colour was developed and read in the usual way.

Volatile ammonium salts. Since the protein was finally eluted with ammonium buffers the preparation was tested for bound volatile ammonium salts by incubation with 50% saturated K₂CO₃ in a Conway microdiffusion unit.

Sialic acid. This was determined by the method of Warren (1959), with *N*-acetylneuraminic acid (L. Light and Co. Ltd., Colnbrook, Bucks) as standard.

Tryptophan. This was determined from the extinctions at 282 mµ and 291 mµ, the wavelengths of the avidin maximum and fine-structure peak respectively. The extinctions were corrected (1) for background absorption, by a linear extrapolation from the 340–360 mµ region, and (2) for tyrosine absorption by using the tyrosine content obtained from the amino acid analysis. These corrections amounted to about 6% at 282 mµ and 3% at 291 mµ. The tryptophan extinction coefficients were assumed to be the same as for free tryptophan at its maximum (279 mµ, 5500) and fine-structure peak (288 mµ, 4570). In this way the shift of the tryptophan spectrum due to combination in the protein is automatically corrected for. The results from the two wavelengths agreed to within 4% and an average of the two values was taken.

Free thiol groups. These were determined by spectrophotometric titration with *p*-chloromercuribenzoate, both in the presence and in the absence of 50 mM-sodium dodecylsulphonate (Boyer, 1954).

RESULTS

Adsorption of avidin by carboxymethylcellulose

The object was to carry out the adsorption at the highest pH consistent with good recovery of the avidin in order to diminish contamination by less-basic proteins. Batch adsorptions from egg white diluted with an equal volume of water showed that 1.5 g. of carboxymethylcellulose/100 ml. of diluted egg white at pH 7 was sufficient to adsorb 80% of the biotin-binding activity. Attempts to use a more efficient column procedure failed owing to clogging of the column by the viscous solution. The carboxymethylcellulose batches with adsorbed proteins were packed into a column and the material was eluted with salt concentration gradients. Ammonium carbonate was initially chosen on account of its volatility and this proved fortunate since it eluted the avidin before most of the other proteins. Ammonium acetate did not possess this specificity and gave much less active avidin preparations.

Preliminary large-scale purification

First carboxymethylcellulose column. Egg white (20 l.) was gently homogenized in 500 ml. batches with equal volumes of deionized water. A small precipitate was removed by centrifuging at 3000 *g* for 30 min. Carboxymethylcellulose filter-cake equivalent to 150 g. dry wt. was stirred into the turbid supernatant (40 l.) and the pH was adjusted to 7.0 with 3*N*-sulphuric acid. After 15 min. of stirring the carboxymethylcellulose was allowed to settle, the supernatant was siphoned off and a second 150 g. batch of carboxymethylcellulose was added to it. The second supernatant was siphoned off and the adsorption repeated once more. The combined sediments (3 × 4 l.) were centrifuged at 1500 *g* for 1 min. and the carboxymethylcellulose was filtered without paper on large Buchner funnels with little or no suction. It was then washed on the funnel with several litres of 50 mM-ammonium acetate, the filter-cakes were sucked as dry as possible and samples were taken for avidin assay. The supernatant from this stage could be used for the preparation of ovalbumin and ovomucoid. The importance of thorough washing should be emphasized since without it the subsequent column is liable to become clogged. Difficulty was sometimes experienced with the filtration owing to a coating of mucinous material on the carboxymethylcellulose. When this occurred the carboxymethylcellulose was given a preliminary washing with 50 mM-ammonium acetate by decantation and centrifuging, before being transferred to the Buchner funnel. If this was still not satisfactory the pH of the ammonium acetate was raised to 9.0 with ammonia and the washing was repeated, although this may lead to a slight loss of avidin.

The filter-cakes were suspended in 50 mM-ammonium acetate buffer, pH 9 (1 l./200 g. wet wt.). The suspension was degassed and poured carefully into a column (100 cm. × 9 cm.), at the bottom of which fresh carboxymethylcellulose (equivalent to 150 g. dry wt.) had been packed without pressure. Liquid was run out as the suspension was poured in and the column was allowed to settle under gravity. Finally several litres of 50 mM-ammonium acetate, pH 9, were run through under a pressure of a few pounds/in.², which compressed the column to a height of 50–60 cm. (hold-up volume, 2.8 l.) and raised the pH of the effluent to 9. The column was washed successively with 6 l. of 0.3% and 2 l. of 0.4% ammonium carbonate, after which $E_{280}^{1\text{cm.}}$ of the effluent was decreased to 0.03. The proteins were now eluted as shown in Fig. 1. The avidin was eluted in the first peak. A second small peak, probably the basic globulin components described by Rhodes *et al.* (1958), closely overlapped it. The lysozyme was brought off in the third peak as a

concentrated solution (5%) by raising the ammonium carbonate concentration to 3%. It could be crystallized directly by raising the pH of the effluent to 10 with sodium hydroxide and adding sodium chloride (50 g./l.). The specific activity of the first two-thirds of the avidin peak was 6–8 units/mg. (50–70% pure avidin) indicating an approximately 1000-fold purification (Table 1).

Second carboxymethylcellulose column. The pooled avidin fractions (up to 3.5 l. of column effluent) were acidified, degassed and rechromatographed on carboxymethylcellulose as described in the Materials and Methods section (Fig. 2). This was essentially a repetition of the previous column with a much lower loading, and the avidin was now well resolved from most of the contaminating proteins. The specific activity of the avidin was 11–12 units/mg., but there was still inactive material accompanying it which formed a shoulder on the trailing edge of the peak.

Third carboxymethylcellulose column. The fractions containing avidin (Fig. 2: fractions 44–94) were acidified, degassed and rechromatographed in ammonium acetate, pH 9.0 (Fig. 3). In this system

Fig. 1. Large-scale preliminary purification of avidin on a carboxymethylcellulose column (9 cm. × 60 cm.). The column, loaded with 60–70 g. of protein from 600 eggs of unknown origin, was eluted with a stepwise gradient of ammonium carbonate, pH 9.0, and 250 ml. fractions were collected manually. ●, Biotin-binding activity; —, E_{280} .

Table 1. Purification of avidin

Details are given in the text.

	Yield of avidin (mg./egg)	Specific activity (μg. of biotin/mg. of protein)
Whole egg white	1.2	0.005
First adsorption on carboxymethylcellulose	0.8	0.1
First carboxymethylcellulose column	0.6	6–9
Second carboxymethylcellulose column	0.55	12
Third carboxymethylcellulose column	0.5	13.5
Amberlite CG-50 column	—	13.5

most of the impurity emerged before the avidin. The specific activity was constant over most of the peak, except for a slight trail of lower activity. Fractions 60–90 from a batch of 900 eggs were pooled and gave a yield of 420 mg. of freeze-dried protein.

Constituents of purified avidin

Before the conditions for the second and third carboxymethylcellulose columns had been arrived at a large-scale preliminary purification was conducted on 20 l. of egg white by using one carboxymethylcellulose column only (first carboxymethylcellulose column; see Fig. 1). This egg white was obtained from 600 eggs from unknown breeds of hen, and the partially purified material obtained from it, when freeze-dried, had a specific activity of 9 $\mu\text{g./mg.}$ (600 mg.). To purify or fractionate this material further a number of small-scale chromatograms were conducted on 1–10 mg. samples by using carboxymethylcellulose or Amberlite CG-50 columns.

Fig. 2. Purification of avidin on the second carboxymethylcellulose column (6 cm. $\times$ 56 cm.) (see the text). The proteins (3 g.) from a preparation similar to that described in Fig. 1 but from Rhode Island $\times$ Light Sussex hens were eluted with ammonium carbonate, pH 9.0, and 36 ml. fractions were collected. ●, Biotin-binding activity; —, E_{280} .

Fig. 3. Purification of avidin on the third carboxymethylcellulose column (6 cm. $\times$ 40 cm.) (see the text). The proteins (1 g.) from a preparation similar to that described in Fig. 2 were eluted with ammonium acetate, pH 9.0, and 36 ml. fractions were collected. ●, Biotin-binding activity; —, E_{280} .

Rechromatography on carboxymethylcellulose proved to be very convenient for removing inactive material and led to the above method involving three carboxymethylcellulose columns for preparing pure avidin. Although more than one active component was indicated by the spreading of the active peak in some of the carboxymethylcellulose chromatograms (M. D. Melamed & N. M. Green, unpublished work) it was not possible with carboxymethylcellulose chromatography to resolve the different avidins.

With Amberlite CG-50 a number of buffer systems were used at pH values between 9.5 and 10.5 (borate–sodium hydroxide, ethanolamine–hydrochloric acid, carbonate–bicarbonate), and of these borate gave the highest activity and the best resolution of the active components. Borate buffer, pH 10 and I 0.195, was selected as the best system and gave a far better resolution than the carboxymethylcellulose columns, but the peaks were broad and the later ones trailed badly. Introduction of shallow concentration gradients sharpened the peaks without loss of resolution. Moore & Stein (1956) pointed out that the use of salt gradients on columns of Amberlite CG-50 was undesirable, since increasing the cation concentration may affect the pH by displacing protons from the carboxyl groups of the resin. We did not experience any difficulties, possibly because at pH 10 the carboxyl groups are almost completely ionized.

Fig. 4 shows the results of a preparative-scale experiment (250 mg.) conducted on part of the preparation from 600 eggs mentioned above. The two main active components (avidins A and B) emerged with a constant specific activity of 13.5 which was the same as that obtained from the final carboxymethylcellulose column. As this value was consistently the highest activity obtained, we have

Fig. 4. Refractionation of avidin from the first carboxymethylcellulose column (see Fig. 1) on an Amberlite CG-50 column (4 cm. $\times$ 48 cm.). The proteins (250 mg.) were eluted with sodium borate buffer, pH 10 and I 0.195, and 9 ml. fractions were collected. A shallow gradient was introduced after the first two peaks had emerged, to decrease trailing. ●, Biotin-binding activity; —, E_{280} .

assumed it to be the activity of pure avidin. This gives 17 900 g. of protein/mole of biotin, which is about one-third of the lower limit of the published values for the molecular weight of avidin (55 000–70 000) (Fraenkel-Conrat *et al.* 1952*a*) and shows that one molecule of avidin binds three molecules of biotin, rather than two as previously suggested. The third component (avidin *C*) either possessed slightly lower intrinsic activity or was associated with some impurity. It was only obtained in small amount and was not further investigated. The amino acid composition of the fractions *A* and *B* (Table 2) are considered in detail below. Fraction *B* contained slightly more arginine and lysine than fraction *A*, which might explain its firmer retention by the column. The biotin-binding capacities of these different avidins were almost identical, so for

a study of the nature of the binding they were not separated. Avidin prepared by threefold chromatography on carboxymethylcellulose has been used for all the experiments of this series of papers. Although Amberlite CG-50 columns gave good resolution they were much more troublesome to use and the final yield of avidin was low. About 75 % of the total protein was eluted from the column, but much of this became insoluble during the concentration and dialysis of the fractions. Only 35 % of the activity was recovered as freeze-dried avidin in the experiment shown in Fig. 4.

If the amino acid differences between fractions *A* and *B* were genetic in origin, the homogeneity should be improved by using eggs from a uniform population of hens. Further preparations were therefore conducted from eggs of a single breed. To

Table 2. *Composition of avidin preparations*

Details are given in the text. Air-dried preparations contain 8–9 % of moisture; all values in the Table, however, are given on a dry weight basis (110° *in vacuo*). The no. of residues/3 biotin residues are given to 2 significant figures; 3 moles of biotin are equivalent to 53 000 g. of avidin D.31.

Avidin preparation	D.31		<i>A</i>		<i>B</i>	
Total N (%)	15.7		15.3			
$E_{280}^{0.1\%}$	1.57		1.56			
Biotin bound (μ g./mg. of avidin)	13.8		13.5		13.3	
	g./100 g.	No. of residues/ 3 biotin residues	g./100 g.	No. of residues/ 3 biotin residues	g./100 g.	No. of residues/ 3 biotin residues
Lysine	8.03	29	8.25	30	9.0	33
Histidine	0.97	3.3	0.94	3.3	1.02	3.5
Arginine	8.40	26	8.6	27	9.6	30
Aspartic acid	11.74	47	11.7	47	11.6	47
Threonine	14.53*	65*	15.1†	68†	14.8†	67†
Serine	5.74*	29*	5.68†	29†	5.55†	28†
Glutamine	9.76	35	9.05	33	9.0	33
Proline	1.58	7.3	1.52	7.1	1.33	6.2
Glycine	5.13	36	5.04	36	5.06	36
Alanine	2.73	17	2.80	17	2.89	17
$\frac{1}{2}$ Cystine (direct)†	0.86*	3.8*	0.85†	3.8†	0.80†	3.6†
$\frac{1}{2}$ Cystine (as cysteic acid)	4.75	21	—	—	—	—
Valine	5.20	24	4.96	23	5.0	23
Methionine	1.87	6.7	1.89	6.8	1.67	6.0
Isoleucine	6.35	26	6.20	25	6.22	26
Leucine	5.75	23	5.70	23	5.73	23
Tyrosine	1.15	3.4	—§	—	1.30	3.8
Phenylalanine	7.00	23	6.96	23	6.52	21
Tryptophan	5.500	14	5.4	14	5.4	14
Hexosamine	3.83	9.1	4.0	10	4.0	10
Hexose¶	5.80	17	—	—	—	—
Amides	—	46	—	—	—	—
Phosphorus	—	—	0.07	1	0.05	1
Total residues	462		463**		465**	
Mean residue weight	119		120		—	
N recovered	103.5%		—		—	
Weight recovered	100.4%		—		—	

* Corrected for destruction, by linear extrapolation from 24 and 72 hr. hydrolysis values.

† Corrected for destruction by using rates found for the D.31 preparation.

‡ Not included in the totals.

§ Overlapped by glucosamine.

|| Calculated as *N*-acetylglucosamine (see the text).

¶ Calculated as mannose (see the text).

** By assuming tyrosine, mannose and cystine to be the same as for the D.31 preparation.

select the most suitable breed the avidin content of individual eggs was determined. This varied considerably between eggs of a single breed (s.d. about 27%). By taking batches of six eggs for each breed the avidin content varied even more from one breed to another, 0.7–2.2 mg./egg (s.d. 40%). With such large variations six eggs was too small a sample and our results did not appear to be a reliable guide to the average contents of large batches. Two large preparations of avidin (D.26 and D.31) were prepared by threefold carboxymethylcellulose chromatography from Sykes hybrid and from Rhode Island $\times$ Light Sussex hybrid hens respectively. Neither strain gave a particularly good yield, though the products were more homogeneous on Amberlite CG-50 (for example D.31 avidin; see Fig. 5) than that obtained from eggs of unknown origin (Fig. 4). Nevertheless, the D.31 avidin which had given a symmetrical peak on carboxymethylcellulose (Fig. 3) showed two active components on Amberlite CG-50 (Fig. 5), which, from their mobilities, were of the 'A' and 'B' types. The slightly lower activity of the second component suggests the presence of a small amount of impurity in the preparation. The greater homogeneity of the D.31 avidin was confirmed by using carboxymethylcellulose columns. The avidin from the eggs of unknown origin had given two active peaks and a long trail of activity when chromatographed under the conditions of Fig. 3 (M. D. Melamed & N. M. Green, unpublished work).

Care must be taken in the selection of methods of concentrating avidin solutions. One preparation was concentrated by pervaporation before freeze-drying. A current of air at room temperature was blown over the solution in dialysis sacs for about 4 hr. Although this was done in the dark, to prevent any photo-oxidation, the absorption spectrum of the product was changed. The extinction at 282 $m\mu$ was lower, and that at 260 $m\mu$ much higher, than

that of the original material. About 25% of the biotin-binding activity was lost. Some of the tryptophan residues in avidin must therefore be unusually sensitive to oxidation, even in the dark. The spectral and biotin-binding properties of this modified avidin are considered in more detail by Green (1963b).

Analytical results

The analytical results are shown in Table 2. Molecular weights were calculated from the biotin bound, by assuming three biotin-binding sites/molecule of protein. The amount of bound biotin found in the preparations after denaturation amounted to only 1% of the total biotin-binding capacity and was not taken into account in the calculations. Although the protein was isolated from ammonia buffers, less than 0.05% of volatile ammonia was found (cf. Chibnall, Mangan & Rees, 1958).

After hydrolysis in 4N-hydrochloric acid at 100° for various times the avidin gave a Rondle & Morgan (1955) chromophore with an absorption spectrum characteristic of hexosamine (Crumpton, 1959). The colour yield was approximately constant over a range of preliminary hydrolysis times extending from 3 to 8 hr. as reported for glucosamine (Johansen *et al.* 1960; Marshall & Neuberger, 1960). The hexosamine was not characterized further and was calculated as *N*-acetylglucosamine. There was less than 0.1 mole of sialic acid/mole of protein. The hexose was calculated as mannose in accordance with the results of Fränkel-Conrat, Snell & Ducay (1952b), who showed this to be the only non-nitrogenous hexose present. The amino acid composition was similar to that obtained by Fränkel-Conrat *et al.* (1952b) with less pure avidin and microbiological assay methods.

The absorption spectrum of avidin showed a maximum at 282 $m\mu$ and a well-resolved tryptophan fine-structure peak at 291 $m\mu$. Both features were shifted 3 $m\mu$ to the red relative to their position in the spectrum of free tryptophan. The whole spectrum is given by Green (1963b).

DISCUSSION

Avidin and its associated proteins emerged from carboxymethylcellulose columns at lower ionic strengths with ammonium carbonate (Fig. 2) than with ammonium acetate (Fig. 3) at the same pH. This may be due to formation of carbamino groups on the protein by transfer of carbon dioxide from carbamate to amino groups of lysine resulting in a decreased positive charge on the protein. Avidin was more affected than its associated impurities. For example, in ammonium acetate (Fig. 3) the impurity emerged before avidin, whereas in ammonium carbonate (Fig. 2) the avidin was eluted

Fig. 5. Refractionation of avidin from the third carboxymethylcellulose column (see Fig. 3) on an Amberlite CG-50 column (1 cm. $\times$ 20 cm.). The proteins (6 mg.) were eluted with sodium borate buffer, pH 10.0, and 0.5 ml. fractions were collected. ●, Biotin-binding activity; —, E_{280} .

first. Comparison with the behaviour of another associated protein in the same buffer systems (M. D. Melamed & N. M. Green, unpublished work) confirmed the preferential elution of avidin by ammonium carbonate.

It was surprising that a pH as high as 10 was required to elute avidin from Amberlite CG-50 columns, since lysozyme, which is even more basic, can be satisfactorily chromatographed at pH 7.2 in 0.2M-phosphate buffers (Tallan & Stein, 1953). This may have been due in part to the much higher molecular weight of avidin and hence the greater net charge. However, this is not consistent with the behaviour on carboxymethylcellulose, where the avidin was eluted before the lysozyme. The strong adsorption on Amberlite CG-50 may also be correlated with the trailing of the peaks and the poor recoveries of avidin from these columns.

The chromatographic heterogeneity of avidin is due to differences in amino acid content. The total number of amino acid residues in fractions A and B was the same within the limits of error. The differences between the two fractions involved several amino acids (arginine, lysine, threonine, serine, isoleucine, methionine, proline and phenylalanine) and it does not seem that any simple genetic hypothesis can explain them. This is not surprising in view of the large number of hens from which the avidin was obtained. It is possible that both fractions are themselves heterogeneous, but that these heterogeneities involved only uncharged amino acids and did not affect mobility on Amberlite CG-50 columns. The composition of avidin D.31 resembled fraction A rather than fraction B, which supports the impression obtained from the Amberlite CG-50 chromatogram (Fig. 5), where the major component corresponded to the first peak of Fig. 4. There was no evidence for the presence of nucleic acid in our preparations, as judged by the absorption spectrum (Green, 1963b) or the phosphorus content.

The agreement between our values for amino acid content and those of Fraenkel-Conrat *et al.* (1952b) is surprisingly good, considering the relatively low avidin content of their preparations (75%, allowing for 10% of moisture and including 10% of avidin-biotin complex which they showed to be present). Their analytical values were consistently 10–20% lower than ours; moreover, 12% of the dry weight was not accounted for. Our recoveries were essentially complete. If the discrepancy were due to some non-protein impurity in their preparation, this would account both for their low amino acid results and for the close agreement with our values for the relative amounts of the different amino acids.

The cystine values given in the Table require some comment. Fraenkel-Conrat *et al.* (1952b)

found very little cystine and concluded that it was probably absent from the pure protein. Direct determinations from the chromatograms of 6N-hydrochloric acid digests by the method of Moore & Stein (Crumpton & Wilkinson, 1963) yielded approximately four half-cystine residues/mole of protein after allowing for destruction by linear extrapolation to zero time from the values for 24 and 72 hr. hydrolyses. The cysteine acid contents of separate runs on digests of performic acid-oxidized protein (Moore, 1963), however, were 21–22 residues/mole. It is likely that the rapid initial rate of destruction of cystine on hydrolysis is due to the high tryptophan and carbohydrate contents of avidin.

The chromatographic and analytical evidence suggests that our preparations contained over 90% of active protein and less than 1% of avidin-biotin complex. The value of three for the number of binding sites/molecule is probably correlated with the three N-terminal alanine residues found by Fraenkel-Conrat & Porter (1952) and suggests the presence of three peptide chains in the molecule. The data on molecular weight in guanidine hydrochloride (Green, 1963c) support the idea of three dissociable sub-units. If the sub-units were identical the molar amino acid composition should be exactly divisible by three. This is clearly not so and the discrepancies (e.g. 3.4 tyrosine, 3.3 histidine, 6.7 methionine, 7.3 proline) are probably too large to be explicable solely in terms of several closely related avidin species in the D.31 preparation.

SUMMARY

1. A simple large-scale purification of avidin from fresh egg white by gradient elution from carboxymethylcellulose has been devised.
2. Two or three active components of similar biotin-binding activity were shown to be present. Two of these were separated on columns of Amberlite CG-50, and found to differ slightly in their amino acid composition, suggesting that the differences may be genetic in origin.
3. The most active preparations bound 13.8 μ g. of biotin/mg. of avidin, corresponding to an equivalent weight of 17 700. This shows that each molecule of avidin can bind three molecules of biotin.
4. The composition of avidin was completely accounted for in terms of amino acid and carbohydrate residues.

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Avidin

3. THE NATURE OF THE BIOTIN-BINDING SITE

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(Received 19 March 1963)

Green (1963a) and Melamed & Green (1963) have shown that each molecule of avidin binds three molecules of biotin and that the complex has a low dissociation constant of about 10^{-15} M. The present paper is concerned with the nature of the binding site.

Previous work on this subject has been confined to (1) microbiological studies by Wright and his co-workers on the binding of biotin analogues (for a summary see György, 1954), and (2) studies of the effect of chemical modification of avidin on its activity (Fraenkel-Conrat, Snell & Ducay, 1952b). The first group of workers showed that the ureido ring of biotin is of great importance for firm binding, but that substitution of the carboxyl group or modification of the thiophan ring has a relatively small effect. They also showed that of the available stereoisomers of D-biotin only DL-epiallobiotin, epimeric at C₄, is bound by avidin. Some of these findings have now been confirmed and extended by more exact measurements of dissociation constants, particularly of weakly bound analogues such as urea and ethyleneurea (imidazolid-2-one). Previous evidence for binding of the latter compounds had

been provided by experiments of Fraenkel-Conrat *et al.* (1952b) which showed that they stabilized avidin against acid or alkaline denaturation. The experiments with chemical modifications were relatively inconclusive in that, though many of the reagents produced slight inactivation, only prolonged esterification, oxidation with hydrogen peroxide-Fe²⁺ ions and treatment with formaldehyde in the presence of an amide led to more than 60% inactivation. The sensitivity to hydrogen peroxide is probably due to the same groups that are responsible for sensitivity to photo-oxidation (György, Rose & Tomarelli, 1942), and both this and the inactivation by formaldehyde could have been due to modification of tryptophan residues. However, this point only became clear from spectroscopic and chemical evidence presented in a preliminary note (Green, 1962). In this it was shown that binding of biotin produces a red shift of the tryptophan absorption bands of avidin which can readily be detected by the resultant difference spectrum. The large difference extinction coefficient at 233 mμ (25 000/mole of biotin bound) provides a useful method for the determination of avidin or biotin by spectrophotometric titration. Biotin analogues, even distantly related ones, gave similar but smaller spectral shifts that have now

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