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Avidin

1. THE USE OF [^{14}C]BIOTIN FOR KINETIC STUDIES AND FOR ASSAY

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The recent demonstration of the role of biotin in a number of carboxylation reactions has led to a renewed interest in the coenzyme function of this vitamin. Avidin, the biotin-binding protein from egg white, has been found to inhibit many of the carboxylating systems and has been widely used in the characterization of biotin-containing enzymes. Although avidin has been considerably purified and its mode of action studied in some detail (Fraenkel-Conrat, Snell & Ducay, 1952), pure preparations have not been conveniently available and the nature of the biotin-binding site has remained obscure, so that reinvestigation of this protein appeared desirable. The assay of avidin has hitherto been based on the sensitive but time-consuming microbiological assay of biotin. A rapid method employing radioactive biotin has therefore been devised. Preliminary kinetic measurements were made on the reaction of avidin with biotin to establish the best conditions for the determination and these limited results are also presented here.

MATERIALS AND METHODS

[^{14}C]*Carbonyl chloride.* ^{14}CO (1 mc; 0·17 m-mole) was supplied by The Radiochemical Centre, Amersham, Bucks., in a tube with a break seal. This tube was sealed to a male B14 joint to which a test tube containing chlorine could be attached. The desired partial pressure of dry redistilled chlorine (0·41 m-mole) was transferred to this test tube on a vacuum line. The chlorine was frozen out with liquid air, and dry nitrogen admitted to the apparatus. The cooled chlorine tube was quickly attached to the CO tube, evacuated (10^{-3} mm.) through a side arm and sealed off. The seal was broken with a glass-covered steel ball that had been retained by a magnet in a second side arm. The apparatus was irradiated for 90 min. with nine 60 w tungsten lamps. The $^{14}COCl_2$ and excess of Cl_2 were condensed in the test tube with liquid air and transferred to a vacuum line. The condensed gases were distilled into a tube containing amalgamated copper turnings and allowed to react for 1 hr. to remove all excess of chlorine. The $^{14}COCl_2$ was distilled back to the original tube together with a small amount of mercury which formed a mirror. This tube was cooled to $-78°$ at which temperature the carbonyl chloride could be distilled into a third tube, cooled in liquid air. Dry nitrogen was admitted to the apparatus, the tube was removed from the vacuum line and the $^{14}COCl_2$ was dissolved in 1 ml. of ice-cold anhydrous chloroform.

D-[2′-^{14}C]*Biotin.* The sulphate of δ-(3,4-diaminothiophan-2-yl)pentanoic acid was prepared from D-biotin by hydrolysis with baryta (Hofmann, Melville & Du Vigneaud, 1941) and 150 mg. was esterified with methanolic HCl. The methyl ester dihydrochloride was converted quantitatively into the free diamino ester by running a solution in anhydrous methanol through a column (6 cm. × 0·8 cm.) of Dowex 2 (OH^- form; 200 mesh) in anhydrous methanol. The methanolic solution was evaporated to dryness *in vacuo*. The gum was redissolved in 1–2 ml. of anhydrous chloroform and again evaporated to dryness *in vacuo*. This was repeated once more. The diamino ester (80 mg.; 0·42 m-mole) began to crystallize on the walls of the flask and was used immediately to avoid losses by self-condensation. It was dissolved in 2 ml. of chloroform and added to the solution of $^{14}COCl_2$ described above. After standing overnight the solution was transferred to a flask, evaporated to dryness and the biotin methyl ester hydrolysed with 1·5 ml. of 1·6 N-NaOH. After warming (at 40°) a cloudy solution was obtained which was kept for 3 hr. at room temperature before the slight precipitate was centrifuged down. The supernatant and washings were concentrated to 1–2 ml. and conc. HCl (0·3 ml.) was added slowly. The biotin crystallized and was filtered and washed with water. It was recrystallized from hot water and freed from a minor radioactive impurity by chromatography on a column (1·6 cm. × 20 cm.) of Dowex 1 (formate form; 200–400 mesh) in aq. 50% (v/v) ethanol. The biotin was eluted as a single sharp

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peak (Fig. 1) with the gradient obtained by running formic acid [6M in 50% (v/v) ethanol] into a constant-volume mixing chamber containing 50% (v/v) ethanol (50 ml.). A small amount (less than 1%) of radioactive impurity emerged with the solvent front. The fractions containing biotin were evaporated to dryness *in vacuo* and recrystallized from hot water. The yield was 24 mg., and the specific activity $14{\cdot}8 \times 10^6$ counts/min./mg. Carrier biotin was added to the residual liquors from the various stages of the purification, and the chromatography and recrystallization were repeated (yield, 32 mg.; specific activity, $2{\cdot}3 \times 10^6$ counts/min./mg.).

The less active sample was freeze-dried in storage tubes that were then sealed *in vacuo*. The more active biotin was dissolved in 70% (v/v) ethanol and applied to seven strips (56 cm. × 1 cm.) of Whatman no. 1 paper, by running the strips through a trough containing the biotin solution. The strips containing 52 μg. of biotin/cm.^{2} were dried in air and sealed in glass tubes *in vacuo*. This method of storage diminished loss by self-irradiation.

Even after 2 years of storage 99·3% of the radioactivity of this biotin was rendered non-diffusible by avidin. The diffusible material (0·7%) was assayed for biotin with avidin, as described below: 85% of it was not bound, showing that there was about 0·6% of radioactive impurity in the biotin. For most purposes this could be ignored.

Determination of radioactivity. In preliminary experiments 0·2–0·4 ml. of a biotin-containing solution plus 0·1 ml. of 0·2% cetyltrimethylammonium bromide were evaporated on 2 cm.^{2} polythene planchets in an oven at 60°. The biotin was then counted at infinite thinness with an end-window Geiger counter. In subsequent experiments a Nuclear–Chicago Corp. automatic gas-flow counter with a Micromil window was employed. Samples of up to 1 ml. were dried down in the centre of the 4 cm.^{2} aluminium planchets and counted. All the radioactivity values obtained by the first method were multiplied by 4·4 to bring them on to the same scale as those from the gas-flow counter.

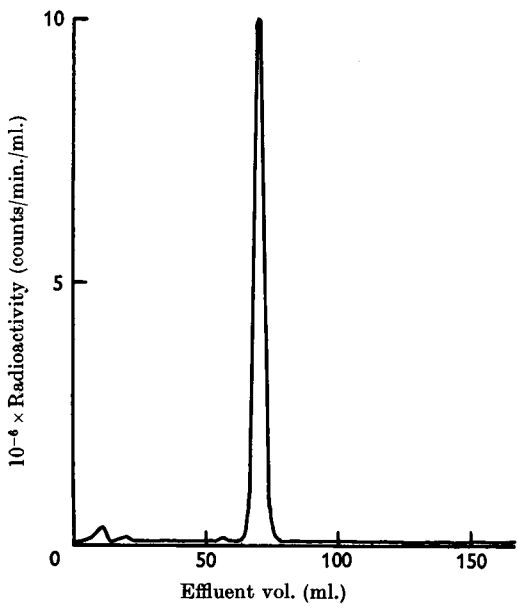

Fig. 1. Elution of [2′-^{14}C]biotin from Dowex 2 (formate form) with a formic acid gradient as described in the text. Fractions of 2·1 ml. were collected.

Avidin. This was purified by chromatography on carboxymethylcellulose as described by Melamed & Green (1963). The assay method was worked out by using approx. 30% pure material, but the kinetic results quoted were obtained with pure avidin.

Radioactive avidin–biotin complex. A slight excess of [^{14}C]biotin was added to a solution of avidin (1 ml.) which was then run through a column (0·9 cm. × 17 cm.) of Sephadex G-25 in 10 mM-ammonium acetate. The protein peak was well separated from the free biotin and was used without further treatment.

Carboxymethylcellulose. Whatman carboxymethylcellulose (0·7 m-equiv. of carboxyl groups/g.) was washed several times by decantation to remove fines. It was then suspended in water at about 60° for 1 hr., resuspended in cold water, packed into a column and washed with water for 48 hr. In the experiments on the kinetics of the avidin–biotin combination, carboxymethylcellulose prepared by the method of Peterson & Sober (1956) (kindly supplied by Miss E. M. Press) was used after washing as described above.

Adsorption of avidin on carboxymethylcellulose. In most of the experiments described in the present paper the adsorption was carried out as a batch process in small test tubes that had been treated with silicone to prevent the carboxymethylcellulose from adhering to the walls. The desired amount of carboxymethylcellulose was obtained by sampling from a suspension in an appropriate buffer with a tuberculin syringe without a needle. The dry weight of carboxymethylcellulose obtained was reproducible within ±10% (S.D.), which was sufficiently accurate for these experiments. Usually the carboxymethylcellulose was spun down and measurements were made on the supernatant. When the rate of adsorption of the avidin–biotin complex was determined the suspension was filtered rapidly with suction at known time-intervals after mixing equal volumes (0·5 ml.) of a suspension of carboxymethylcellulose with a solution of the complex. The amount of complex in the filtrate was determined from its radioactivity.

Rate of combination of avidin and biotin. It was necessary to use a concentration of 10 μmM to decrease the rate to a measurable level. Stock solutions of avidin and [^{14}C]biotin (20 μmM) in 2 mN-acetic acid adjusted to pH 5·0 with ammonia were kept at 25° and transferred immediately before use to a pair of similar 10 ml. syringes. These syringes were emptied in about 1·5 sec. via a 1 ml. glass mixing chamber into a flask maintained at 25° which was stirred magnetically. The reaction was stopped after a few seconds by diluting the [^{14}C]biotin with a 100-fold excess of unlabelled biotin (0·2 ml. delivered from a third syringe). Avidin–biotin complex was removed from the reaction mixture by running it through a column (0·9 cm. × 1·5 cm.) of carboxymethylcellulose (20 ml./hr.). The first 3 ml. of effluent was evaporated to dryness in an oven at 70°, dissolved in 1 ml. of 10 mN-ammonia, plated and counted. It was established in separate experiments that the amount of [^{14}C]biotin displaced from the complex during the separation procedure was negligible.

Rate of exchange of free biotin with avidin-bound biotin. The reaction mixture (5·0 ml.), prepared in 2 mN-ammonia adjusted to pH 5·0 with acetic acid, contained 5·4 μg. of [^{14}C]biotin as avidin–biotin complex and 78 μg. of free biotin. It was incubated at 25°, in the presence of a little toluene to prevent bacterial growth, and duplicate 0·1 ml. samples were removed at intervals. The radioactive avidin–biotin complex was removed on columns (0·5 cm. × 0·6 cm.) of carboxymethylcellulose and the displaced [^{14}C]biotin was washed through on to 4 cm.^{2} aluminium planchets with 1 ml. of water. The planchets were treated with a thin smear of silicone grease round the edges so that the liquid sample remained in the centre.

Determination of avidin activity. Method 1: use of carboxymethylcellulose. At pH 5 both avidin and avidin–biotin complex were quantitatively adsorbed on carboxymethylcellulose at ionic strengths below 0·4. A suspension of washed carboxymethylcellulose (10–12 mg./ml.) was prepared in 0·1 M-pyridine–acetate buffer, pH 5·0 [2 N-acetic acid (25 ml.) and pyridine (4·25 ml.) diluted to 500 ml.], containing radioactive biotin at a concentration of 1000–2000 counts/min./ml. Samples (1 ml.) of this suspension were dispensed from a tuberculin syringe without a needle into small test tubes containing the avidin samples (0·2 ml.). After standing with occasional shaking for 2–3 min. the carboxymethylcellulose was centrifuged down, and the excess of [^{14}C]biotin was determined by plating and counting a sample (0·2–0·4 ml.) of the supernatant. The specific activity of the avidin was expressed as μg. of biotin bound/ml. of avidin solution of E_{280} 1·0.

Method 2: ultrafiltration. Twelve dialysis sacs (0·5 cm. × 5 cm.) were attached to a pressure manifold, each via a small glass T-piece and a rubber sleeve. Avidin and excess of biotin were pipetted into the sacs through one arm of the T-piece, which was then closed with a screw clip. A 1 l. air reservoir was attached, the pressure was raised to 10 lb./in.^{2} and the system was closed off. Ultrafiltrate was collected in small test tubes for 2–3 hr. Samples were then plated and counted, and the biotin bound to avidin was determined by difference. This method was only used for rough estimations.

RESULTS

Synthesis of [2′-^{14}C]*biotin.* [^{14}C]Biotin had previously been prepared from the sulphate of the diamino acid resulting from the alkaline hydrolysis of biotin (Melville, Pierce & Partridge, 1949). When this compound was treated with [^{14}C]-carbonyl chloride in the presence of 0·1 N-sodium hydroxide, the ureido ring was re-formed and a 30 % radiochemical yield of D-biotin was obtained. Trial experiments gave lower yields than this, presumably owing to hydrolysis of the carbonyl chloride, and it was decided to attempt the synthesis in a non-aqueous solvent. The only good solvent found for the diamino acid was dimethylformamide, and many experiments were carried out, usually in the presence of a slight excess of pyridine or triethylamine, but only very low yields of biotin were obtained. It was then observed that the addition of larger amounts of carbonyl chloride to dimethylformamide gave a white precipitate that reacted vigorously with water. This appeared to be analogous to the quaternary-ammonium-type complexes formed between acid halides and tertiary bases (Adkins & Thompson, 1949) with one of the alkyl groups replaced by a formyl group. Such a formyl group would become an active acylating agent so that there would be a number of different possible reactions between the complex and the diamino acid, leading to low yields of biotin. Good yields were eventually obtained by using the ester of the diamino acid which dissolved readily in chloroform. An excess of the ester was employed to take up the hydrogen chloride formed in the reaction.

Avidin assay. The principle of the assay method was to add an excess of [^{14}C]biotin to the sample of avidin and to determine the excess after adsorbing the avidin–biotin complex on carboxymethylcellulose. To establish the best condition for the assay and to obtain some idea of its limitations, measurements of the following were made: (1) the concentration of free avidin–biotin complex in equilibrium with the complex adsorbed on carboxymethylcellulose; (2) the rate of adsorption of avidin–biotin complex by the carboxymethylcellulose; (3) the rate of combination of avidin and biotin; (4) the rate of exchange of free biotin with avidin-bound biotin.

The measurements were all made at pH 5·0, where avidin was firmly bound by carboxymethylcellulose up to ionic strengths of 0·4. Pyridine–acetate buffer (0·1 M) was normally used since its volatility eliminated the necessity for self-absorption corrections, but pyridine was replaced by ammonia in the kinetic experiments on the avidin–biotin reaction.

The apparent concentration of avidin–biotin complex in equilibrium with avidin–biotin complex adsorbed on carboxymethylcellulose was dependent on the previous history of the carboxymethylcellulose. With inadequately washed material much larger quantities of avidin–biotin complex remained in the supernatant, which suggested that a slightly soluble component of the carboxymethylcellulose was forming a complex with the avidin–biotin complex and preventing its adsorption. When thoroughly washed Whatman carboxymethylcellulose was used about 10 % of the avidin–biotin complex remained unbound (Fig. 2, lower curve), but if an aqueous extract of unwashed carboxymethylcellulose (0·5 mg./ml.) was added to the system, much larger amounts of avidin–biotin complex remained in the supernatant (Fig. 2, upper curve). The results of Fig. 2 show that the soluble carboxymethylcellulose material, which in this experiment accounted for 2–3 % of the dry weight, bound more than 50 % of the avidin–biotin complex. Its solubility was possibly due to a larger-than-average content of carboxymethyl groups

(Peterson & Sober, 1956) and this, taken together with its solubility, could explain its affinity for avidin which appears to be so much greater than that of the insoluble carboxymethylcellulose.

Carboxymethylcellulose prepared by the method of Peterson & Sober (1956) contained much less of this soluble material, and only 3% of the avidin–biotin complex remained in solution. It was therefore used in the experiments on the kinetics of the avidin–biotin reaction, where an efficient adsorption was particularly desirable.

The adsorption of the radioactive avidin–biotin complex was rapid. The time for half adsorption was 3 sec. and the process was 95% complete in 20 sec., in 0·1 M-pyridine–acetate buffer, pH 5·0.

Rate of combination of avidin with biotin. Preliminary experiments in 0·1 M-pyridine–acetate buffer gave a second-order rate constant of 5×10^5 M^{-1} sec.$^{-1}$, corresponding to a half-time of 2 sec. in 1 μM solution. When ammonium acetate buffers were used the reaction was much faster and could only be followed by diluting the reaction mixture to 10 μmM. Because of the extreme dilution about 20% of the avidin–biotin complex was not retained on the carboxymethylcellulose columns used to separate it from unchanged biotin, and it was necessary to correct the results accordingly. Errors also arose from the low radioactivities being measured and from the mixing time being an appreciable proportion of the reaction time. The results are of only moderate accuracy as indicated by the second-order plot shown in Fig. 3. The rate constants calculated from two experiments were $6{\cdot}5 \times 10^7$ and $8{\cdot}2 \times 10^7$ M^{-1} sec.$^{-1}$.

Rate of exchange of free biotin with avidin-bound biotin. In theory this should be a first-order process in which the rate-determining step would be the dissociation of the avidin–biotin complex. The first-order plot in Fig. 4 shows that about 4% of the bound [^{14}C]biotin exchanged relatively rapidly ($k = 10^{-6}$ sec.$^{-1}$), whereas the rate constant for the next 12% of exchange was only 9×10^{-8} sec.$^{-1}$. Other experiments showed that up to 20% of the [^{14}C]biotin could exchange at this rate, but the incubation times required to demonstrate more complete exchange were prohibitively long ($t_{\frac{1}{2}} = 90$ days).

An approximate value of the dissociation constant of a single binding site of 10^{-15} M was obtained from the ratio of the rate constants for combination and exchange. From this an approximate free energy of binding of 20 kcal./mole of biotin bound was calculated.

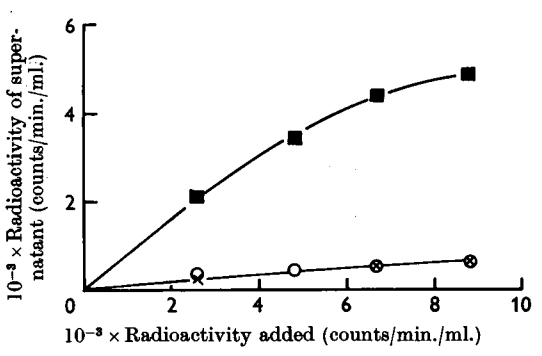

Fig. 2. Adsorption of radioactive avidin–biotin complex by carboxymethylcellulose at pH 5·0. Each tube contained 2 ml. of pyridine–acetate buffer, pH 5·0, 25 mg. of carboxymethylcellulose and the amount of radioactive avidin–biotin complex indicated. ○, 20 mM-Buffer; ×, 0·11 M-buffer; ■, 20 mM-buffer and 0·3 mg. of 'soluble carboxymethylcellulose'/ml.

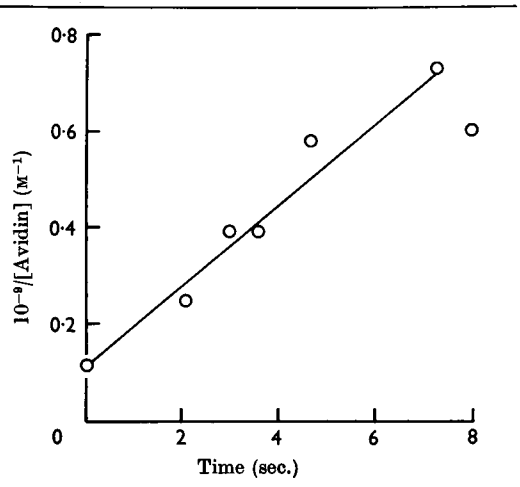

Fig. 3. Kinetics of avidin–biotin combination in ammonium acetate buffer, pH 5·0 and I 0·002, at 25°. The plot is second order. The avidin and [^{14}C]biotin (20 μmM) were mixed rapidly and allowed to react for a known time before quenching of the reaction with an excess of unlabelled biotin. The unchanged [^{14}C]biotin was separated and determined as described in the Materials and Methods section.

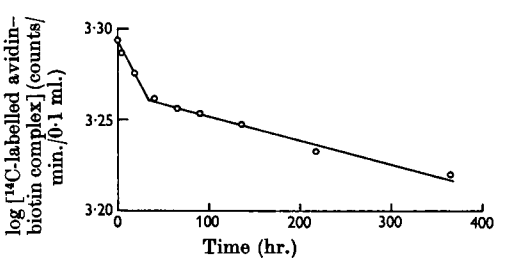

Fig. 4. Kinetics of the exchange between unlabelled biotin and [^{14}C]biotin bound to avidin, in ammonium acetate buffer, pH 5·0 and I 0·002, at 25°. Samples were removed from the reaction mixture at known time-intervals, and the displaced radioactive biotin was separated and determined as described in the Materials and Methods section.

Determination of avidin. In the light of the above results the technique described in the Materials and Methods section was devised and employed to determine the free biotin left after the addition of increasing amounts of avidin to a series of similar samples of [^{14}C]biotin (Fig. 5). The titration departs from linearity in the neighbourhood of the equivalence point, because of the incomplete adsorption of avidin–biotin complex by the carboxymethylcellulose. The true equivalence point is given by the intersection of the two linear portions of the curve. This titration procedure was used if an accurate determination of biotin-binding activity was required, but for most purposes (e.g. assay of column fractions) one or two duplicate points were taken by using, as far as possible, the middle region of the curve. Results were often unreliable when more than 75% of the biotin was bound, on account of slight variations in the amount of avidin–biotin complex left in solution by different carboxymethylcellulose samples. The S.D. of a series of 12 replicate determinations at 60% neutralization, which had been counted for 4000 counts, was ±3·5%. With biotin of specific activity 14 800 counts/min./μg., 0·01 μg. of biotin or 1 μg. of avidin could readily be detected, and 5 μg. of avidin could be determined with the specified accuracy.

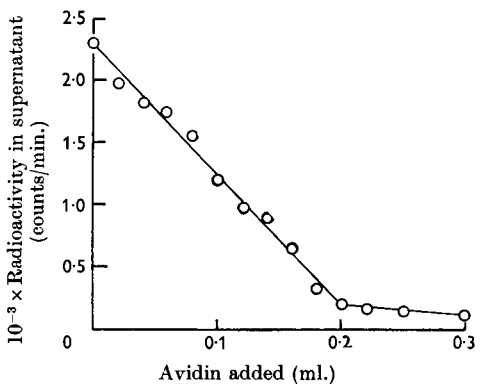

Fig. 5. Titration of [^{14}C]biotin with avidin. Avidin was added to 1 ml. of a suspension of carboxymethylcellulose in 0·1 M-pyridine-acetate, pH 5·0, containing [^{14}C]biotin (2300 counts/min.). The excess of biotin was determined after centrifuging down the carboxymethylcellulose.

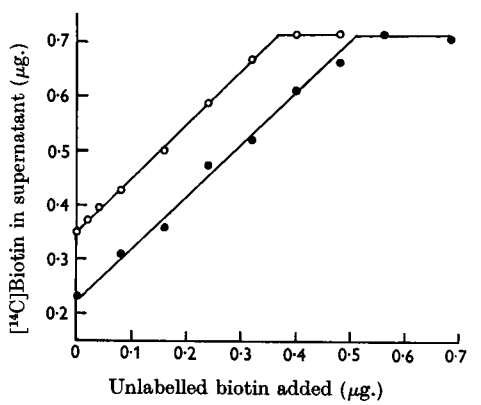

Fig. 6. Determination of unlabelled biotin. The biotin was added in increasing amounts to a series of tubes containing an excess of avidin. The excess was determined by the addition of a carboxymethylcellulose suspension containing [^{14}C]biotin (0·7 μg.) in excess of the total avidin present. ○, 40 μg. of avidin; ●, 57 μg. of avidin.

The volume of avidin solution taken was usually only a small proportion of the volume of carboxymethylcellulose suspension so that large variations of pH and salt concentration could be tolerated. In crude avidin preparations the inert proteins present gave rise to counting errors due to self-absorption. This could be avoided by adsorbing the avidin on carboxymethylcellulose in the absence of [^{14}C]biotin, washing twice with 0·1 M-pyridine–acetate buffer by centrifuging and finally adding the [^{14}C]biotin. The prior adsorption of the avidin on carboxymethylcellulose had no effect on its biotin-binding capacity. An alternative approach that was sometimes used under these circumstances was to determine the excess of [^{14}C]biotin in an ultrafiltrate as described in the Materials and Methods section.

Determination of unlabelled biotin. Because of the extremely slow exchange of free biotin with bound biotin described above, it was possible to extend the method to the determination of unlabelled biotin by a back-titration procedure (Fig. 6). The unlabelled biotin prevents the binding of an equivalent amount of [^{14}C]biotin, confirming the slow exchange rate. Biotin can clearly be determined by this method in systems where it is the only substance bound by avidin. Further work on the specificity of the method would be required before it could be extended to biological systems, where much of the biotin is present in bound forms.

DISCUSSION

The rate constant for combination of avidin with biotin ($k = 7 \times 10^7$ M^{-1} sec.$^{-1}$) is of the same order as those for combination of a number of enzymes with their substrates (e.g. fumarase, 10^7 M^{-1} sec.$^{-1}$; Alberty & Peirce, 1957) and as that for the reaction of carboxyhaem with globin (5×10^8 M^{-1} sec.$^{-1}$; Gibson & Antonini, 1960). The high rate of these reactions suggests that one of the rate-limiting factors is the diffusion of the small molecule to the binding site. Alberty & Hammes (1958) have used the theory of Smoluchowski to calculate the maximum rate of such a diffusion-controlled process. By

assuming that the small molecule diffuses into a hemispherical sink on the surface of the protein, they showed that $k = 7{\cdot}5 \times 10^{20} Dr$, where D is the sum of the diffusion coefficients of the reacting species and r the critical distance of approach for combination to occur. By assuming the diffusion constant of biotin to be approx. 6×10^{-6} cm.^{2}/sec. [diffusion constants of sufficient accuracy for these calculations were obtained from published data (Cohn & Edsall, 1943) on molecules of similar size and shape to those involved here; biotin was assumed to be similar to tryptophan; protein diffusion constants were estimated by assuming the molecules to be approximately spherical] and r to be 10 Å (Alberty & Hammes, 1958), the rate constant for uncharged molecules would be 5×10^8 M^{-1} sec.$^{-1}$. Since at pH 5 biotin (pK 5·1) has about 0·5 unit of negative charge, and avidin a large though diffusely distributed positive charge, the maximum theoretical rate will be somewhat larger than this (Alberty & Hammes, 1958). It is therefore unlikely that more than one encounter in ten leads to reaction.

From the data of Kaziro, Leone & Ochoa (1960) the rate constant for the reaction between avidin and the biotinyl-enzyme, 'propionyl carboxylase' (propionyl-coenzyme A carboxylase, EC 6.4.1.3), can be calculated to be 3×10^6 M^{-1} sec.$^{-1}$. The theoretical maximum value assuming a diffusion-controlled reaction between neutral molecules would be approx. 8×10^7 M^{-1} sec.$^{-1}$ [assuming that the reaction takes place between the avidin and an enzyme unit containing a single biotin (mol.wt. 175 000; $D = 4 \times 10^{-7}$ cm.^{2} sec.$^{-1}$)]. From this rough estimate one can conclude that encounters between avidin and enzyme-bound biotin are of the same order of efficiency as those between avidin and free biotin. This suggests that the biotin in biotinyl-enzymes is freely exposed on the enzyme surface as has been suggested on different grounds by Lynen *et al.* (1961). This would be in harmony with the probable mechanism of action of these enzymes, since the biotin must be sufficiently mobile to shuttle between two active centres, namely an ATP–Mg^{2+}–HCO_3^- centre and a substrate-carboxylating centre.

The interpretation of the data on the exchange rate is not completely unequivocal since the process was not followed to completion. Green (1963*b*) has shown that the biotin combines randomly at three sites on the avidin molecule, and it is therefore likely that the three sites are similar and that all the biotin can exchange with the same rate constant (9×10^{-8} sec.$^{-1}$). The 5% of radioactivity that exchanged more rapidly might be due either to a contaminant of the [^{14}C]biotin or to the presence of some slightly denatured avidin. In view of the extensive purification of the biotin by crystallization and ion-exchange chromatography, the first possibility seems unlikely, although some decomposition during storage due to auto-irradiation cannot be ruled out. The exchange is so slow that for most purposes the avidin–biotin combination can be regarded as irreversible, and this is in harmony with the results on the irreversible inhibition of biotinyl-enzymes by avidin (see Wakil, 1962, for references).

Halenz, Feng, Hegre & Lane (1962) have reported that, by using high concentrations of biotin (1 mM; 200-fold excess with respect to avidin), the avidin inhibition of 'propionyl carboxylase' could be partially reversed (25%). Apart from this the only reported example of a reversible inhibition has been the avidin inhibition of the exchange of [^{14}C]-propionyl-CoA into methylmalonyl-CoA, also catalysed by 'propionyl carboxylase' (Friedman & Stern, 1961; Halenz & Lane, 1961). The addition of biotin to give a concentration of 1 mM completely reversed the inhibition, whereas lower concentrations produced partial reversal. Since it is unlikely from the foregoing considerations that the avidin was displaced from the inhibited enzyme by the added free biotin it seems possible that the exchange reaction can be directly catalysed by the added biotin, bound reversibly in the neighbourhood of the site for the carboxylation of propionyl-CoA. This would be in harmony with the dependence of the reversal on biotin concentration. The differential effect of biotin on the exchange reaction and on the overall carboxylation would be due to the loss of carboxylated biotin from the enzyme before it could shuttle between the substrate-carboxylation site and the ATP–Mg^{2+}–HCO_3^- site. This interpretation is related to that put forward by Lynen *et al.* (1961) for the carboxylation of free biotin by methylcrotonyl-coenzyme A carboxylase.

The dissociation constant calculated from the ratio of the rate constants is considerably lower than estimates of this quantity by Launer & Fraenkel-Conrat (1951). They made an approximate estimate of 5×10^{-13} M from the amount of avidin–biotin complex required to support the growth of *Saccharomyces cerevisiae*. An equilibrium-dialysis method with radioactive biotin gave a value of 10^{-11} M, but the interpretation of this result was complicated by the presence of radioactive impurities in the biotin. Moreover, the possibility discussed above that avidin preparations may contain a proportion of molecules that release their biotin more readily than the bulk of the material might account for the high apparent dissociation constants obtained by this method.

The great advantage of the assay method lies in its simplicity and rapidity. Unfortunately it requires radioactive biotin which is not at present

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

SUMMARY

1. [2′-^{14}C]Biotin has been synthesized from ^{14}CO in 70% radiochemical yield.

2. A rapid and simple assay for avidin has been worked out, based on the determination of the excess of [^{14}C]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 (7×10^7 M^{-1} 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 (9×10^{-8} 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.

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Avidin

2. PURIFICATION AND COMPOSITION

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Avidin preparations of high activity were obtained by Fraenkel-Conrat, Snell & Ducay (1952*a*). 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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