Three N-acyl derivatives of biotinylethylenediamine were prepared: I, biotinylamidethyoethyl-3-(3-[125I]iodo-4-hydroxyphenyl)propionamide; II, biotinylamidothioethyl-[^3H]acetamide; and III, biotinylamidothioethyl-3-(3,5-[125I]diido-4-hydroxyphenyl)propionamide. Each compound was combined with a large excess of avidin, yielding 1:1 molar complexes. Aside from a small fraction of each complex that dissociated more rapidly, the dissociation half-lives of these complexes were: I, 41 days; II, 4.4 days; and III, 148 days. The iodo- (mono or di) hydroxypropynylpropionyl moieties of I and III, therefore, contribute significantly to the binding strength of these compounds toward avidin. We also formed 4:1 complexes of I, II, and III with avidin (compound in excess), each of which exhibited biphasic dissociation, with initial half-lives of 4, 3.2, and 24 days, respectively. Thus, I or especially III potentially can be used as a sensitive tracer in quantitative studies with avidin.

The avidin-biotin system (Green, 1975) continues to emerge as a useful molecular binding tool in work with biological molecules, as has been reviewed (Wilchek & Bayer, 1984; Bayer & Wilchek, 1980). This system is attractive largely because avidin is a small stable protein that binds four biotin molecules, and avidin is somewhat hydrophilic and easily conjugated onto other molecules giving products that complex with avidin. The avidin-biotin system has been useful, for example, in both qualitative and quantitative studies of membrane receptors (Wilchek and Bayer, 1984) and in increasing the sensitivity of detecting DNA probes (Singer and Ward, 1982).

To facilitate some of the studies and applications of the avidin-biotin system, it would be useful to possess a radioactive derivative of biotin that could be detected with high sensitivity. An 125I-labeled Bolton-Hunter derivative is a logical choice due to the commercial availability of the 125I-labeled Bolton-Hunter reagent and the high specific activity of 125I.

Here we present such a derivative of biotin, in which ethylenediamine is used as a spacer group bridging the biotin and 125I-labeled Bolton-Hunter moiety. Because the resulting biotin derivative was found to bind unusually strongly to avidin, we also prepared and similarly tested corresponding [125I]diido-Bolton-Hunter and [3H]acetyl derivatives of biotin.

**EXPERIMENTAL PROCEDURES**

Materials—Affinity purified avidin (14.6 units/mg) and (+)biotin were from Sigma. 125I-Labeled Bolton-Hunter reagent (NEX-120), [125I]diido-Bolton-Hunter reagent (NEX-120H), and [3H]acetic anhydride (NET-018A) were from Du Pont-New England Nuclear. Dimethylformamide (DMF) and ethylenediamine were redistilled prior to use. All other chemicals were of the highest purity available.

Conjugation—HABA was made on a gradient system equipped with a variable wavelength detector. Gamma radiation from 125I was monitored externally through Tygon tubing by a sodium iodide scintillation crystal and a ratemeter from Ludlum Instruments. TLC was done on a silica gel plate from Analtech. For detection of radioactivity after cutting, plastic-backed silica from Eastman Kodak was used. Solvent A was 1-butanol:acetic acid:water (70:7:10, v/v/v), and solvent B was 1-butanol:acetic acid:water:3-mercaptopentanol (70:7:10:2.8, v/v/v/v). Biotin and its derivatives were specifically stained with p-(dimethylamino)cinnamaldehyde as described (McCormick and Roth, 1970). Separation of free from bound radioactive biotin derivative in the avidin binding assays was accomplished by spotting 3–5 μl on an ITLC strip (1.2 x 9 cm from Gelman), allowing it to dry for 3–4 min, and then developing it in 0.15 M NaCl, ethanol (96.5, v/v).

**Biotinylethylenediamine**—Biotin (1 g, 4.09 mmol) was dissolved in 14–20 ml of DMF by heating and magnetic stirring. The solution was cooled to ambient temperature, and N,N' -carbonyldimidazole (973 mg, 5 mmol) in 1 ml of DMF was added giving a white precipitate within 10 min. After an additional 50 min, ethylenediamine (1.07 ml, 16 mmol) in 10 ml of DMF was added, and stirring was continued for 2 h. The semisolid residue after rotary evaporation was dissolved in 50 ml of refluxing methanol, filtered (paper), and again taken to dryness. Imidazole was removed by extraction with chloroform (5 x 100 ml). The residue was dissolved in a little water, acidified to pH 3 with 1 M HCl, and taken to near dryness. Crystallization from methanol gave 1.1 g of product (85% yield). This product was a single spot (Rf = 0.25, positive by iodine vapor, ninhydrin, and p-(dimethylamino)cinnamaldehyde by TLC (solvent A). m.p. = 129 °C; [H NMR (D2O) 3.2–3.6 (4H, multiplet, –NCH2CH2–). IR 1525 cm⁻¹ (–CONH–).

**Biotinylamidoethyl-3-(3-[125I]iodo-4-hydroxyphenyl)propionamide** (I)—125I-Labeled Bolton-Hunter reagent (2 μCi in 0.1 ml of benzene) (Boltan and Hunter, 1973) was taken to dryness by vacuum. Biotinylethylenediamine (100 μg) in 30 μl of 0.5 M potassium phosphate buffer, pH 8.0, was added, and the reaction was kept at 4 °C for 30 min. After dilution with 0.2 ml of water, the entire mixture was purified by HPLC on a Waters C-18 column eluted with 0.1% acetic acid:acetonitrile (80:20, v/v) for 30 min. Unlabeled biotinylethylenediamine eluted at the void volume, hydroxylated Bolton-Hunter reagent eluted at 12 min, and the desired conjugate eluted as a single peak, detected by both UV and radioactivity, at 26 min. Its radiochemical yield was

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 consisted almost 80%. This product was diluted to 50 μCi/ml with 50% ethanol containing 0.01 M β-mercaptoethanol. In the absence of the latter reagent, the product gradually oxidized to α and β sulfoxides. TLC was done on a 250-μm plate using solvent system B which contained β-mercaptoethanol. Initial radiochemical purity of the product was 0.75% and was achieved in 30 min of storage at −10 or 24 °C. Assuming that this compound was both chemically and radiochemically pure as indicated by both HPLC and TLC, then its specific activity was 2200 Ci/mmol, the same as the 125I-labeled Bolton-Hunter reagent.

Biotinylamidoethylacetamide (Nonradioactive II)—Acetylation of biotinylamidoethylamine was done by modifying the method for peptides (Riordan and Vallee, 1967). Biotinylthylenediamine HCl (50 mg, 0.155 mmol) was dissolved in 5 ml of 0.5 M sodium acetate buffer and acetylated with acetic anhydride (158 mg, 1.55 mmol) overnight at 4 °C. The acetylated product was extracted with 1-butanol, dried by rotary evaporation, redissolved in 0.1% acetic acid, and purified by HPLC using a Rainin Dynamax C-18 column (21.4 mm × 25 cm) eluted at 5 ml/min. Injection was made into acetonitrile, 0.1% acetic acid in water (10:90, v/v), and this mobile phase was immediately changed to 15:85, v/v. The product eluted at 30 min and was lyophilized. It was a single spot by TLC (250 μm) with solvent A (Rf = 0.31) positive by iodine vapor and p-(dimethylamino)cinnamaldehyde. m.p. = 218–219 °C. Mass spectrum, m/z = 328. NMR (CD3OD, D2O) δ 3.31–3.34 (4H, multiplet, –NHCH2CH2–), 1.98 (3H, singlet, –CH3).

Biotinylamidoethyl-[3H]acetamide (II)—[3H]Acetic anhydride (1 ml) was added to −78 °C in a breakseal tube. Biotinylthylenediamine HCl (644 μg, 2 μmol) in 0.64 ml of 0.5 M sodium acetate buffer was added, and the acetylation proceeded at ambient temperature for 30 min. Ethanol (5 ml) containing 0.05 M β-mercaptoethanol was added, and the reaction mixture was stored at −20 °C until purification. An aliquot (400 μl) was taken to dryness by vacuum and redissolved in 0.1% acetic acid. HPLC was done using a Waters C-18 column eluted with 0.1% aqueous acetic acid at 0.15 ml/min with solvent B (Rf = 0.31). The specific radioactivity of the product was determined to be 24 Ci/mmol by the HABA avidin binding assay (Green, 1970).

After 3 weeks at −20 °C, the purity was still >95% by TLC.

Radioactive and Nonradioactive I and III—Chloramine T (45 mg, 0.16 mmol) and NaCl (24 mg, 0.18 mmol) were reacted in 1 ml sodium phosphate buffer, pH 8.0 (10.2 ml) for 5 s at room temperature. To this oxidized iodine solution was added succinimidyl 3-(p-hydroxyphenyl)propionamide (42 mg, 0.16 mmol) dissolved in 0.65 ml of ethyl acetate and 2.1 ml of methanol. After 10 s the reaction was quenched with sodium metabisulfite (30 mg, 0.16 mmol) in 0.1 M sodium phosphate, pH 8.0 (0.45 ml). Immediately biotinylthylenediamine (52 mg, 0.16 mmol) in 1.9 ml of 0.1 M sodium phosphate, pH 8.0, was added. After 1 h, the reaction was purified by semi-preparative HPLC in three aliquots. A Dynamax C-18 column (21.4 mm × 25 cm) eluted at 5 ml/min with solvent A (Rf = 0.05 M β-mercaptoethanol) was used to elute the radioactivity. The nitride (Riordan and Vallee, 1970) was added to each tube. Since a quantitative end point was desired, the protein concentration of the avidin stock solution was measured by the HABA assay (Green, 1975), and the biotin binding site concentration was measured by the HABA assay (Green, 1970). Avidin/tube based on UV was 3.45 pmol, and biotin binding sites measured by the HABA assay were 3.40 pmol/tube. Increasing amounts of I to avidin was determined by ITLC as described above.

Dissociation Kinetics for I:Avi d In a 1:1 Complex—Into duplicate 12 × 75-mm polystyrene test tubes were added 0.25 ml of 0.05 M potassium phosphate, pH 7.4, 0.15 M NaCl, 0.05% lysozyme, radiiodinated tracer (0.5 μCi, 0.23 μmol), and avidin (2.5 pg, 37 pmol). After incubation for 20 min, biotin (2 μg, 85 pmol) was added, and the temperature was maintained at either 4 or 20 °C. A temperature-controlled recirculating water bath (Haake) was used to maintain 20 °C. Free from bound tracer was determined at subsequent time intervals by spotting 4 μl from each sample on an ITLC strip (1 × 6 cm) and developing in 0.15 M NaCl, ethanol (95:5, v/v). The avidin and any bound I stayed at the origin while any free I migrated to the solvent front. The strip was cut in half, and each section was counted in a γ counter. Percent bound was calculated as (origin cpm/(origin + solvent front cpm)) × 100.

Dissociation Kinetics for II:Avi d—This was done as described for I except that a 1:2 complex was formed by adding another equivalent of avidin (0.5 μCi, 27 pmol) in 126 μl of buffer (concentrated from the original storage solution) was added to avidin (0.236 μg, 3.45 pmol of monomer) in 300 μl of buffer and incubated at 20 °C for 7 h. Excess biotin (22 μg, 90 nmol) was added at time zero.

Dissociation Kinetics for III:Avi d—II (0.15 μCi, 6.25 nmol) was incubated with excess avidin (1.5 mg, 22 nmol) in 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.4, 0.15 M NaCl, 0.05% lysozyme, 0.05% sodiumoxide for 2 h at ambient temperature. Unlabeled biotin (1.5 mg, 6 μmol) was added, and the solution was kept at 4 or 20 °C. Percent bound was determined as above except 5 μl was spotted, and the radioactivity was determined from the front section of the ITLC strip after overnight drying. In 1.5 ml of ethanol for 1 h to elute the radioactivity. Liquid scintillation fluid (4.5 ml) was added, and the radioactivity was measured (counting efficiency for 1H was 31.9%). To determine the total counts spotted on the ITLC strip, a blank strip (no sample application) was prepared, cut, and soaked in the same ethanol. Five μl of II if added (the same amount as spotted above), followed by the fluid and counting. Percent bound was calculated as (1 – (solvent front cpm/total cpm)) × 100.

Dissociation Kinetics for III:Avi d—II (0.15 μCi, 6.25 nmol) was added to avidin (78 μg, 1.17 nmol) in 600 μl of 0.05 M sodium phosphate buffer, pH 7.4, 0.15 M sodium chloride, 0.05% lysozyme, 0.05% sodiumascorbate, and incubated overnight at ambient temperature. Excess biotin (150 μg, 600 nmol) was added at time zero, and the temperature was controlled at 30 °C. Percent bound was determined as described above for III:Avi d (1:1).

Dissociation Kinetics for I:Avi d—(1:1)—Binding assays were set up as for I with the following changes. Sodium ascrobate (0.05%, w/v) was present in the buffer. III (0.25 μCi, 0.014 pmol) and avidin (1.25 μg, 18.5 pmol) were incubated for 1 h at 24 °C. The temperature was then controlled at 4 or 20 °C, and biotin (21 μg, 86 nmol) was added at time zero. Separation of free from bound was done as described for I.

Dissociation Kinetics for III:Avi d—(1:4)—When 14.2 fmol of III was plated in a polystyrene test tube in the usual phosphate-saline-lysozyme buffer, and avidin (14 fmol intended) was added, no binding was observed. An apparent excess of avidin was added at 20°C to 14 fmol of III. More avidin was added (total 149 fmol intended) until ≥ 0.82% binding of III was achieved. The exact amount of avidin in solution at that point was unknown. After incubating the samples at 24 °C for 20 h,

R. K. Garlick and R. W. Giese, unpublished observations.
50 nmoles of biotin were added to initiate dissociation, and percent bound was determined as above.

*Denaturation of Avidin:*—The above 1:1 avidin:III complex (100 µl) was combined with 200 µl of ethanolic acetic acid (90:20, v/v) and heated to 70-74 °C for 1 h. Percent binding was determined by ITLC as previously described.

**Dissociation Kinetics for [3H]Biotin:Avidin (1:1)—[3H]Biotin (3.75 µCi, 44 Ci/mmol) in 0.25 ml of 0.02 M β-mercaptoethanol:ethanol (80:20, v/v) in a glass test tube was taken to dryness by rotary evaporation. The [3H]biotin was redissolved in 0.65 ml of 0.05 M sodium phosphate, pH 7.4, 0.15 M sodium chloride, 0.06% lysozyme, 0.06% sodium azide. A 100-µl portion containing 0.625 µCi (14.2 pmol) of [3H]biotin was added to 9.4 µg (140 pmol) of avidin dissolved in the same buffer in a polystyrene test tube. This was incubated at ambient temperature for 1 h, and then excess biotin (3.4 µg, 14 nmol) was added. The temperature was kept at 20 °C, and separation of free from bound was done as described for II.

**RESULTS**

The scheme that we followed to prepare three radiolabeled derivatives of biotin is shown in Fig. 1. First biotin was converted to biotinyl-ethylene diammine. From this latter compound was then prepared 125I-labeled Bolton-Hunter conjugate I, a corresponding 125I-acetyl conjugate II, and a corresponding 125I-diiodo-Bolton-Hunter product III.

**Preparation of I—** I was synthesized by preparing biotinyl-ethylene diammine and conjugating it with an 125I-labeled Bolton-Hunter reagent as shown in Fig. 1. Radiochemical yields after HPLC were consistently high, usually above 80%.

**Binding of I to Avidin**—The stoichiometry of I binding to avidin was determined by titrating avidin (3.6 pmol of avidin monomer by UV; 3.4 pmol of biotin binding capacity by HABA) with I (data not shown). A sharp end point was observed at 3.45 pmol of I, revealing a molar binding stoichiometry, as expected, of 4:1 for the binding of I to avidin.

To determine the dissociation rate for I complexed to avidin, a large excess of biotin was added (2000-fold molar excess over avidin binding sites) at time zero, and separation of free from avidin-bound I was done by ITLC as a function of time. Free I migrated with the solvent front, and bound I remained at the origin. This technique gave a rapid separation (3-4 min) with low blanks (1-2%; see Footnote a in Table I).

Original assay buffer contained bovine serum albumin as a protein carrier, but a poor precision prompted a switch to 0.05% lysozyme which overcame this problem.

The dissociation rate and corresponding half-life (t₁/₂) of I complexed to avidin as a function of temperature is shown in Fig. 2 and in Table I. For a 1:1 complex of I with avidin obtained by incubating I with a 160-fold molar excess of avidin (640 molar excess of avidin binding sites) the dissociation is basically monophasic at both 20 and 4 °C, pH 7.4. (There is a small fraction of more rapid or "anomalous" dissociation at the outset that is discussed below.) The half-lives for the dissociations at these two temperatures are 41 and 380 days, respectively. At 20 °C, pH 8.5, the half-life is 18 days (Table I).

Biphasic dissociation is seen when a 4:1 complex of I to avidin is formed (1.3 molar excess of I over avidin binding sites) at 20 °C, pH 7.4 (Fig. 2). The initial dissociation rate for the 4:1 complex corresponds to a half-life of 4 days, which is 10 times faster than for the corresponding 1:1 complex.

The second slower rate matches that of the 1:1 complex, and extrapolating this latter rate back to the ordinate for the 4:1 complex gives an intercept at 50% binding. This demonstrates that both the 1:1 and 2:1 complexes of I to avidin share the same dissociation half-lives. Presumably the residual sites on avidin are fully occupied by biotin shortly after its addition in these experiments.

**Compound II**—To better understand the contribution of the 125I-labeled Bolton-Hunter group to the tight binding of I to avidin (in terms of dissociation), we prepared and similarly tested the corresponding 125I-acetyl compound, structure II in Fig. 1. The dissociation half-lives at pH 7.4 of II as a 1:1 complex with avidin at 20 and 4 °C are 4.4 and 51 days, respectively, as shown in Fig. 3 and cited in Table I. Thus, II dissociates nearly 10 times faster than does I at both of the corresponding temperatures, demonstrating that the 4-hydroxy-3-iodobenzyl moiety of I contributes significantly to the binding of I to avidin. Two other conditions were evaluated as well, as indicated in Table I; the half-life was 3.2 days at 24 °C, pH 7.4, and 4.8 days at 20 °C, pH 8.5.

Shown as an inset in Fig. 3 is the initial dissociation of II from avidin when a 4:1 complex is formed. The half-life of 3.2 days for the latter complex is only slightly shorter than that of the corresponding 1:1 complex. This contrasts with the 10-fold difference in the dissociation rates for the analogous complexes between avidin and I.

**Compound III**—To further explore the region near the binding site on avidin that interacts favorably with the

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*Fig. 1. Synthesis. a, N,N'-carbonyldimidazole, ethylenediamine, DMF; b, acetic anhydride, 0.5 M sodium acetate; c, [125I]iodo-Bolton-Hunter reagent, 0.5 M sodium phosphate, pH 8.0; d, [125I]iodo-Bolton-Hunter reagent, 0.5 M sodium phosphate buffer, pH 8.0.*
ritaries arising from decay catastrophe of IZI as a function of which is shown in Fig. 1, a second iodine atom is present, replacing an 0.18 mM (41). Necessary to account for the formation of radioactive impu-

The dissociation of a 1:1 complex of avidin and III is shown in Fig. 4. Since III was doubly labeled with 125I, it was necessary to account for the formation of radioactive impurities arising from decay catastrophe of III as a function of time. This was done both experimentally (TLC) and by cal-

FIG. 4. Dissociation of III:avidin as a 1:1 complex at 4 °C (●) and at 20 °C (△). Inset, 4:1 complex at 20 °C (●). Concentration of unlabeled biotin: 8 mM (1:1), 0.97 mM (4:1).

As seen in Fig. 4, and summarized in Table I, III complexed to avidin at 20 °C, pH 7.4, dissociates with a half-life of 148 days after correcting for decay. Thus, III, in this sense, binds 3.6 times stronger to avidin than does I. The half-life was unchanged at pH 8.5 (150 days) but decreased slightly at pH 5.0 (113 days).

At 4 °C the dissociation half-life of III was too slow to measure accurately, given the complication of decay catastrophe. Nevertheless, the relative slopes of the 4 °C dissociation line and the decay catastrophe line for III by least squares regression analysis suggest a dissociation half-life of 6.4 years.

We confirmed that neither III nor any of its radioactive decay products covalently bound to avidin during this experiment by extracting all of the counts from a 60-day-old complex of avidin and III with ethanolic acetic acid.

We also measured the dissociation half-life of a 4:1 complex of III and avidin. Once again only an estimate could be made because of experimental difficulties. The problem in this case was that the intense radioactivity and small amount of III limited the amount of avidin that could be used. As seen in the inset of Fig. 4, there is evidence for biphasic kinetics, and the initial shorter half-life after correcting for decay appears to be about 24 days.

| Table I  |
| Dissociation of biotin derivatives complexed to avidin |

| Compound | t\text{diss,}^{4:1} | t\text{diss,}^{1:1} | 1:1 complexes with anomalous dissociation |
|----------|-----------------|-----------------|-----------------------------------------|
| (mono-iodo) pH 7.4, 20 °C | 4 | 41 | 3 |
| pH 8.5, 20 °C | 18 | 5 |
| pH 7.4, 4 °C | 380 | 2 |
| (acetyl) pH 7.4, 20 °C | 3.2 | 4.4 | 18 (1.0)* |
| pH 7.4, 24 °C | 3.2 | 16 |
| pH 8.5, 20 °C | 4.8 | 8 |
| pH 7.4, 4 °C | 51 | 5 |
| (diiodo) pH 7.4, 20 °C | 24 | 148 | 5 |
| pH 8.5, 20 °C | 150 | 5 |
| pH 5.0, 20 °C | 113 | 6 |
| pH 7.4, 4 °C | 4-6 years | 1 |
| Biotin | 473 | 8 (26)* |

*The radiolabeled biotin derivative or biotin plus avidin were preincubated at room temperature from 0.3 to 2 h prior to setting the incubation temperature and adding excess biotin at time zero for the 1:1 complexes where a large excess of avidin was present. For the 4:1 complexes, preincubation was overnight. Percent binding refers to B/Bo × 100, where B is the fraction of total counts (origin and solvent front) found in the origin half of the ITLC strip, and Bo is the same counts when no biotin is added to initiate dissociation. In this calculation there was no correction for the blank in which 1-2% of the tracer is found in the origin half of the ITLC strip in the absence of avidin. All of the biotin derivatives gave an initial B/Bo × 100 = 99-100% (obtained immediately after addition of excess nonradioactive biotin).

For the 4:1 complexes, the biotin derivative is present in a significant molar excess (see "Experimental Procedures") over the four binding sites on avidin.

For the 1:1 complexes avidin is present in a significant molar excess (see "Experimental Procedures") over the amount of the biotin derivative.

Anomalous dissociation was calculated as q − r where q = initial value of B/Bo × 100 (see Footnote a) and r = the extrapolated value for B/Bo × 100 on the y axis using the predominant slope (later part; least squares linear regression analysis of the subsequent data points) of the dissociation curve.

Dissociation half-life in days for the initial anomalous dissociation.

FIG. 2. Dissociation of an avidin complex of I. ●, 1:1 complex (excess avidin) at 4 °C; △, 1:1 complex at 20 °C; ○, 4:1 complex (excess I) at 20 °C. Concentration of unlabeled biotin: 0.10 mM (1:1), 0.19 mM (4:1).

FIG. 3. Dissociation of II:avidin as a 1:1 complex at 4 °C (●) and at 20 °C (△). Inset, 4:1 complex at 20 °C (●). Concentration of unlabeled biotin: 8 mM (1:1), 0.97 mM (4:1).
The very slow dissociation of I complexed 1:1 with avidin (tₐₙ = 6 weeks at 20 °C, 1.04 year at 4 °C) was not anticipated. Others generally have encountered much weaker binding of monosubstituted biotin derivatives to avidin in the presence of excess avidin. The longest half-life observed by Chignell et al. (1975) for our typical conditions (pH 7.4, 20 °C) is 473 days. As with our biotin derivatives, anomalous dissociation (8.0% of the complex) is observed at the outset, although it is much slower (tₐₙ = 26 days) than for these derivatives.

**DISCUSSION**

The dissociation of I and its complexes is suggestive of such a cleft (Honatko and Williams, 1982). Our observation that II binds to avidin with a half-life 10-fold shorter than that of I confirms that the Bolton-Hunter group of I helps to anchor I to avidin. Whether or not this is due to hydrophobic binding is another matter, however. For example, the Bolton-Hunter group contains an ionizable phenolic OH that might form a hydrogen or salt bond with avidin. The pKₐ of this OH is anticipated to be near 8.8 (Rogoeuci, 1984; Mayberry et al. 1965). II binds just as strongly to avidin at pH 8.5 as at pH 7.4, showing that the overall structure of avidin in this complex is not perturbed by this change in pH. However, I dissociates twice as fast at the higher pH. Thus, the weaker binding to avidin of I at pH 8.5 versus 7.4 is probably due to a specific unfavorable interaction between these species that does not develop between II and avidin with this increase in pH. Apparently either the ionization of I or of a side chain on avidin near the Bolton-Hunter group of I is responsible.

To probe this in more detail, we also determined the binding strength of avidin for III, a diiodo version of I anticipated to have a phenolic pKₐ near 6.8–7.1 (Rogoeuci, 1984; Mayberry et al., 1965). At pH 7.4 the binding of III to avidin (tₐₙ = 148 days) is not only 3.6-fold stronger than that of I but is unchanged when the pH is increased to 8.5. Decreasing the pH from 7.4 to 5.0, which passes through the phenolic pKₐ of III, slightly weakens the binding of III to avidin (the tₐₙ falls from 148 to 113 days). This latter result is probably due to a general effect on pH on the avidin binding site rather than protonation of III, since avidin also binds biotin 2-fold weaker at pH 5 versus 7 (Green, 1975).

The simplest explanation for the differences in the dissociation half-lives for the binding of I, II, and III to avidin, including effects of pH, is that the Bolton-Hunter group, monoiodo or diiodo, basically makes a favorable contact with an accessory hydrophobic binding site on the avidin. The phenolic hydroxyl does not play much of a role unless it is ionized and unshielded as in I at pH 8.5, where it slightly weakens the hydrophobic interaction.

Model building shows that the distance between the biotin moiety and the iodine atom(s) of I or III can easily be made to match the corresponding distance between the biotin moiety and the side chain of Val-22 or Tyr-23 in [biocytin]²⁵ACTH₁₋₂₆amide, which has a flexible alkyly spacer between the biotin and insulin, has a half-life at 25 °C of 76 days as a 1:1 complex (Finn and Hofmann, 1985). Moreover, the distance is also small and flexible, and yet their complexes with avidin dissociate rapidly. Thus, additional factors may play a role in the strong binding of [biocytin]²⁵ACTH₁₋₂₆amide to avidin (see below).

We postulated that I binds strongly to avidin not only because I avoids repulsive interactions with avidin but also because the Bolton-Hunter group (3-iodo-4-hydroxyphenylpropionyl) of I interacts favorably with avidin. In particular, it was attractive to consider a hydrophobic interaction between the latter group and avidin given the structural characteristics of this group. Chignell et al. (1975) proposed that the biotin sites on avidin are located in a hydrophobic cleft. This was based on their observation, using ESR, that nonpolar spin labels conjugated to biotin are highly immobilized when these conjugates are complexed with avidin. The secondary structure of avidin is suggestive of such a cleft (Honatko and Williams, 1982).

Anomalous Dissociation — A close examination of the figures showing dissociation of the 1:1 complexes reveals that the data does not extrapolate to an initial value for B/B₀ × 100 of 99–100% (see Footnote a in Table I). Thus, there is an initial more rapid dissociation of some of the biotin derivative complexed to avidin when biotin is added.

**Biotin** — To provide a reference point for our dissociation half-life measurements, we measured the dissociation of a 1:1 complex of avidin and biotin using [³H]biotin. As seen in Fig. 5 and listed in Table I, the half-life for this complex under our typical conditions (pH 7.4, 20 °C) is 473 days. As with our biotin derivatives, anomalous dissociation (8.0% of the complex) is observed at the outset, although it is much slower (tₐₙ = 26 days) than for these derivatives.

**Fig. 5. Dissociation of [³H]biotin as a 1:1 complex with avidin at 20 °C.**

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**Biotin** — To provide a reference point for our dissociation half-life measurements, we measured the dissociation of a 1:1 complex of avidin and biotin using [³H]biotin. As seen in Fig. 5 and listed in Table I, the half-life for this complex under our typical conditions (pH 7.4, 20 °C) is 473 days. As with our biotin derivatives, anomalous dissociation (8.0% of the complex) is observed at the outset, although it is much slower (tₐₙ = 26 days) than for these derivatives.

**DISCUSSION**

The very slow dissociation of I complexed 1:1 with avidin (tₐₙ = 6 weeks at 20 °C, 1.04 year at 4 °C) was not anticipated. Others generally have encountered much weaker binding of monosubstituted biotin derivatives to avidin in the presence of excess avidin. The longest half-life observed by Chignell et al. (1975) for a biotinyl–spinlabel conjugate (4-biotinamido-2,2,6,6-tetramethyl-1-piperidinyloxy) was 15.5 h at 25 °C. The 1:1 complex of N°-biotinylamido-insulin with succinylavidin at 25 °C had a half-life of 2.6 h (Finn and Hofmann, 1985).

However, more in line with our result, [biocytin]²⁵ACTH₁₋₂₆amide binds strongly to succinylavidin; the half-life is 20 days for dissociation of the 1:1 complex at room temperature (Romovec et al., 1983). This enhanced binding was attributed by the authors to the small size and flexibility of this biotinyl peptide. Supporting this concept, N°-biotinylamido(6-biotinamido)hexanoylinsulin, having a flexible alkyly spacer between the biotin and insulin, has a half-life at 25 °C of 76 days as a 1:1 complex (Finn and Hofmann, 1985). Nevertheless, most of the other biotin conjugates cited above are also small and flexible, and yet their complexes with avidin dissociate rapidly. Thus, additional factors may play a role in the strong binding of [biocytin]²⁵ACTH₁₋₂₆amide to avidin (see below).

We postulated that I binds strongly to avidin not only because I avoids repulsive interactions with avidin but also because the Bolton-Hunter group (3-iodo-4-hydroxyphenylpropionyl) of I interacts favorably with avidin. In particular, it was attractive to consider a hydrophobic interaction between the latter group and avidin given the structural characteristics of this group. Chignell et al. (1975) proposed that the biotin sites on avidin are located in a hydrophobic cleft. This was based on their observation, using ESR, that nonpolar spin labels conjugated to biotin are highly immobilized when these conjugates are complexed with avidin. The secondary structure of avidin is suggestive of such a cleft (Honatko and Williams, 1982).
The presence of some higher complexes (e.g. 2:1) in the 1:1 incubation mixture in which adjacent biotin binding sites are both occupied by the biotin derivative, giving a faster dissociation characteristic of a 4:1 complex (see below), does not account either for the anomalous dissociation, since the rate for the latter is still faster in each case than for the 4:1 complex.

We postulate that the anomalous dissociation is due to microheterogeneity of the avidin from the usual pre- and post-translational variations of proteins (Whitaker and Fujimaki, 1980). The carbohydrate of avidin is heterogeneous (Bruch and White, 1982), and either isoleucine or threonine is found at position 34 (DeLange and Huang, 1971). Some of the tryptophan of avidin is associated with the binding site for biotin and is susceptible to oxidation (Green, 1975). Ovalbumin, another protein from egg white, is acetylated, glycosylated, phosphorylated, and has an amino acid replacement (Nisbet et al., 1981). Contaminants that bind noncovalently to avidin also might play a role, e.g. nuclear acid contaminants have been observed (Fraenkel-Conrat et al., 1952). The different amounts of the anomalous dissociation for our biotin derivatives would then reflect differences in their susceptibility to the various types of microheterogeneity.

Two phases of dissociation for 4:1 complexes of avidin and biotin conjugates are usually seen (e.g. Chignell et al., 1975). These authors concluded that the initial phase of the usual biphasic kinetics for 4:1 complexes arises from an unfavorable interaction between the conjugate groups of the biotin derivatives when these derivatives bind to the adjacent biotin sites on avidin. Subsequent 2:1 and 1:1 complexes, in which the derivatives occupy residual nonadjacent sites without crowding, then dissociate more slowly. Consistent with this picture, for our compounds the dissociation rates for the 4:1 and 2:1 complexes are similar only for II, the smallest of our derivatives of biotinylethylenediamine. One of the biotin derivatives examined by Chignell et al. (1975) as a 4:1 complex with avidin also dissociated monophasically.

It is anticipated that future studies and applications of the avidin-biotin system will benefit some of the results reported here and in particular as follows. 1) Because of their tight binding to avidin and their intense radioactivity, the 125I-labeled biotin derivatives of I and III are potentially useful as labels for some studies and applications of the avidin-biotin system. 2) Information is gained concerning the hydrophobic binding characteristics near the biotin binding site on avidin and the anomalous dissociation of biotin and biotin derivatives complexed to avidin. 3) Preliminary x-ray crystallographic analysis of avidin has been reported (Gatti et al., 1984; Finn et al., 1982; Green and Joynson, 1970). Such work should benefit by the availability of nonradioactive forms of I and III.

REFERENCES

Bayer, E. A. & Wilchek, M. (1980) Methods Biochem. Anal. 26, 1-45
Bolton, A. E. & Hunter, W. M. (1973) Biochem. J. 133, 529-531
Bruch, R. C. & White, H. B., III (1982) Biochemistry 21, 5334-5341
Chignell, C. F., Starkweather, D. K. & Sinha, B. K. (1975) J. Biol. Chem. 250, 5622-5630
DeLange, R. J. & Huang, T. S. (1971) J. Biol. Chem. 246, 698-709
Finn, F. M. & Hofmann, H. (1985) Methods Enzymol. 109, 418-445
Fraenkel-Conrat, H., Smell, N. S. & Ducay, E. D. (1952) Arch. Biochem. Biophys. 39, 80-96
Gatti, G., Bolognesi, M., Coda, A., Chiolero, F., Filippini, E. & Malcovati, M. (1984) J. Mol. Biol. 178, 787-789
Green, N. M. (1963) Biochem. J. 89, 585-591
Green, N. M. (1970) Methods Enzymol. 18A, 418-424
Green, N. M. (1975) Adv. Protein Chem. 29, 85-133
Green, N. M. & Joynson, M. A. (1970) Biochem. J. 118, 71-72
Honatko, R. H. & Williams, R. W. (1982) Biochemistry 21, 6201-6205
Mayberry, W. E., Ball, J. E., Berman, M. & Bertoli, D. (1965) Biochemistry 4, 1965-1972
McCormick, D. B. & Roth, J. (1970) Anal. Biochem. 34, 226-236
Nisbet, A. D., Saundry, R. H., Mor, A. J. G., Fothergill, E. A. & Fothergill, J. E. (1981) Eur. J. Biochem. 115, 355-345
Pinn, E., Pahler, A., Saenger, W., Petsko, G. & Green, N. M. (1982) Eur. J. Biochem. 123, 545-546
Riordan, J. F. & Vallee, B. L. (1967) Methods Enzymol. 11, 565-570
Rogovitski, E. R. (1984) Iodine-labeled Plasma Proteins, p. 150, CRC Press, Inc., Boca Raton, FL
Ronovacek, H., Finn, F. F. & Hofmann, K. (1983) Biochemistry 22, 904-909
Singer, R. H. & Ward, D. C. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7331-7335
Whitaker, J. R. & Fujimaki, M. (1980) Chemical Deterioration of Proteins, American Chemical Society, Wash. D.C.
Wilchek, M. & Bayer, E. (1984) Immunol. Today 5, 39-43