

A Reagent for Covalently Attaching Biotin to Proteins via a Cleavable Connector Arm¹

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The preparation of a reagent which can be used to attach biotin to proteins via a cleavable connector arm is described. The reagent, 3-(4-(*N*-biotinoyl-6-aminocaproyloxy)phenyl)propionic acid *N*-hydroxysuccinimide ester (BPE), can acylate amino groups of proteins and other compounds via reaction of the *N*-hydroxysuccinimide ester function. Compounds which have been covalently labeled with BPE can be selectively retrieved (at physiological pH values) from complex mixtures by adsorbing the target compounds on immobilized avidin. These compounds can be released from the avidin by cleavage of the phenyl ester linkage of the connector arm under conditions which don't denature proteins. Insulin was labeled with BPE. The binding of the labeled insulin to immobilized avidin, and its subsequent release by treatment at room temperature with 1 M hydroxylamine at pH 7 was demonstrated.

The highly specific and very strong binding of biotin to avidin ($K_D \sim 10^{-15}$ M; (7)) could provide us with a basis for the design of elegant protocols for the retrieval of compounds from cells and physiological fluids even when the compounds targeted for retrieval are present at very low concentrations. For example, a polypeptide hormone that had been covalently labeled with biotin could be incubated with cells and later retrieved by adsorption on immobilized avidin for studies of hormone catabolism and for isolation of hormone-receptor complexes. Unfortunately, the direct application of this approach is usually precluded by the very low rate constant for the dissociation of biotin from

avidin ($t_{1/2} \sim 200$ days; (7)), and the fact that all of the methods which are known to increase the rate of decomposition of the avidin:biotin complex lead to irreversible protein denaturation. This difficulty has been overcome to some extent by the use of 2-iminobiotin (8, 10). Unlike biotin, 2-iminobiotin interacts with avidin very poorly at low pH values. Thus, proteins labeled with 2-iminobiotin can be eluted from immobilized avidin at pH 4. There are two limitations associated with the use of 2-iminobiotin, however. One is that the pH must be above 9.5 for immobilized avidin to exhibit significant adsorption of 2-iminobiotin-labeled proteins from dilute solutions (10). The other is that avidin binds 2-iminobiotin much less tightly than biotin. For example, the relative affinity of biotin and 2-iminobiotin for avidin is 2.8×10^7 at pH 9.5 and 3.5×10^4 at pH values well above 12 where 2-iminobiotin (pK_a 11.9) exists primarily in its basic form. These considerations suggest that a biotin label might be more useful than

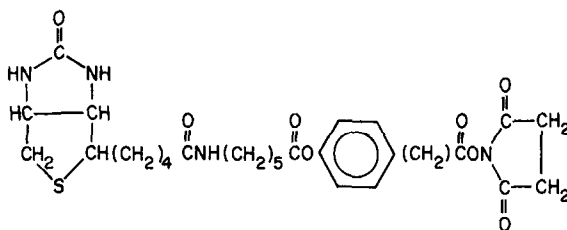
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a 2-iminobiotin label for specific retrieval of proteins and macromolecular complexes of proteins which are unstable in alkaline

solution or are present at very low concentrations (or both). In this work we describe the preparation of a biotin derivative (BPE)⁴ which can be used to link bio-



BPE

tin to polypeptides and other compounds via a cleavable connector arm. After the biotinylated compound has been adsorbed on immobilized avidin under physiological conditions, it can be selectively released from the avidin by breaking the phenyl ester linkage in the connector arm. The cleavable phenyl ester linkage contained in BPE has an advantage over other cleavable linkages commonly used in crosslinking agents, in that it can be broken under mild conditions which do not result in reduction of disulfide bonds or oxidation of glycosyl residues.

MATERIALS AND METHODS

N-Hydroxysuccinimide (NHS) obtained from Aldrich Chemical Company was recrystallized from ethyl acetate. Dimethyl sulfoxide (Me₂SO) was dried over a molecular sieve and trifluoroacetic acid (Aldrich Chemical Co.) was distilled. Triethylamine, dimethylformamide (DMF), isopropanol, and dioxane were distilled from phthalic anhydride, P₂O₅, Ca₂SO₄, and LiAlH₄, respectively.

Benzyl 3-(4-hydroxyphenyl)propionate (I). 3-(4-Hydroxyphenyl)propionic acid (HPPA), 50 g (0.3 mol) as obtained from Aldrich Chemical Company, was converted to the monopotassium salt by dissolving it in 300 ml of 1 N KOH, and removing the water under reduced pressure. The monopotassium salt of HPPA was dissolved in 400 ml of warm 95% ethanol in a round-bottom flask equipped with a reflux condenser. The solution was heated to reflux, and benzyl bromide (32.5 ml, 0.273 mol) in 100 ml of ethanol was added over 1 h with stirring. The reaction mixture was heated under reflux for another 1.5 h, whereupon it was cooled, and the ethanol removed under reduced pressure. The resulting oil was suspended in 1.5 liters of H₂O, neutralized to pH 7 with NaHCO₃, and stirred for 0.5 h. The aqueous suspension was then extracted

three times with ether, and the combined ether extract, was back-extracted with 0.02 N sodium phosphate buffer (pH 7). After the ether extract was dried over sodium sulfate, the ether was removed under reduced pressure, and the resulting oil was distilled under reduced pressure. The fraction which boiled at 170–174°C, 0.03 mm Hg, was collected to yield 46.8 g (68%) of the desired product, an oil (*d* = 1.179) NMR (60 MHz CDCl₃ + 1% TMS) δ 7.28 (5H, s, ArH), δ 6.92 (4H, m, ArH), δ 5.09 (2H, s, ArCH₂O), δ 2.72 (4H, m, CH₂). The product exhibited a change in molar absorptivity accompanying neutralization (with KOH) of the phenolic hydroxyl group which was within 3% of that seen for HPPA ($\Delta\epsilon_{295}$ 2540 cm⁻¹ M⁻¹ in 20% ethanol-water).

N-Benzylloxycarbonyl-6-aminocaproic acid (II). Benzyl chloroformate (48 ml, 0.34 mol) was added slowly (over 1 h) to a vigorously stirred solution of 6-aminocaproic acid (40 g, 0.31 mol) in 38 ml of 4 N NaOH which was cooled in an ice bath, and maintained at pH 9.5–10.5 by addition of 4 N NaOH. After addition of the benzyl chloroformate, the solution was stirred for another 5 min, extracted three times

⁴ Abbreviations used: B-insulin, the derivatives of insulin produced upon modification of insulin and tracer amounts of [¹²⁵I]insulin with excess biotin *N*-hydroxysuccinimide ester; BPE, 3-(4-(biotinoyl-6-aminocaproxyloxy)phenyl)propionic acid *N*-hydroxysuccinimide ester; BPE-insulin, the derivatives of insulin produced upon modification of insulin and tracer amounts of [¹²⁵I]insulin with excess BPE; BSA, bovine serum albumin; DMF, dimethylformamide; HPPA, 3-(4-hydroxyphenyl)propionic acid; NHS, *N*-hydroxysuccinimide; NHS-CM-Sepharose, a derivative of CM-Sepharose wherein the Sepharose-linked carboxylic acid groups have been converted to *N*-hydroxysuccinimide esters; avidin-Sepharose, an immobilized avidin derivative obtained by reacting avidin with NHS-CM-Sepharose; Me, methyl.

with ether, and acidified to pH 1 with concentrated HCl. The resulting precipitate was extracted into ethyl acetate. After drying the extract over Na_2SO_4 , the ethyl acetate was removed under reduced pressure. The solid residue was purified by three recrystallizations from ether:petroleum ether to yield 53.9 g (68%) of the desired product, mp 55°C (lit. $54\text{--}55^\circ\text{C}$) (11) ir (KBr) amide 1732 cm^{-1} , NMR (60 MHz, CDCl_3 + 1% TMS) δ 7.36 (5H, s, ArH), δ 5.08 (2H, s, ArCH_2), δ 3.15, δ 2.30, δ 1.42 (10H, m, CH_2).

Benzyl 3-(4-(N-benzyloxycarbonyl-6-aminocaproxyloxy)phenyl)propionate (III). II (12 g, 45 mmol), I (12 ml, 54 mmol), and POCl_3 (2 ml, 22 mmol) were mixed in a round-bottom flask equipped with a reflux condenser. The reaction mixture was heated in an oil bath at 100°C for 1.25 h, then cooled to room temperature, and extracted with ether by stirring the solution vigorously with three 300-ml portions of ether. The combined ether extract was back-extracted three times with 0.02 N sodium phosphate buffer (pH 7). Ether was removed from the ether extract under reduced pressure. The residue was taken up in benzene and the benzene removed under reduced pressure. This process was repeated once so as to remove traces of water. The resulting residue, 12 g of crude III, was used without further purification. The phenyl ester content of crude III was determined as described below and was 59% of that expected for pure III.

3-(4-(6-Aminocaproxyloxy)phenyl)propionic acid hydrobromide (IV). Crude III (2.49 g containing 2.92 mmol phenyl ester) and anisole (1 ml, 9.2 mmol) were mixed with 9 ml of trifluoroacetic acid, and HBr (g) was bubbled through the reaction mixture. After 1.75 h, (which was found to be the optimum reaction time), the reaction mixture was poured with stirring into 300 ml of ether. The optimum reaction time for removal of the benzyl and benzyloxycarbonyl groups was indicated by the formation of a precipitate approximately 5 min after 50 μl of the reaction mixture had been added to 1 ml of ether. Extended reaction times resulted in decomposition of the desired reaction product. After the reaction mixture had been stirred with the ether for 20 min, the resulting solid was collected, dissolved in ethanol, and precipitated with ether repeatedly to yield 0.46 g (44%) of IV mp $160\text{--}161^\circ\text{C}$. The phenyl ester content (see below) of IV was 100% of that expected for pure IV, NMR (60 Hz, DMSO - d_6 + 1% TMS) δ 1.52 (6H, m, CH_2), δ 2.70 (8H, m, CH_2), δ 7.20 (4H, m, ArH), δ 7.98 (3H, m, NH_3^+).

Biotin N-hydroxysuccinimide ester (V). This compound was synthesized using a procedure similar to that of May *et al.* (9). A solution of biotin (4.01 g, 16.4 mmol) and NHS (2.075 g, 18.0 mmol) in 70 ml of Me_2SO was dried over molecular sieve (4 \AA) for 1.25 h. The molecular sieve was removed, washed with 5 ml of dry Me_2SO , and the wash was combined with

the solution of biotin and NHS. *N,N'*-Dicyclohexylcarbodiimide (3.4 g, 16.5 mmol) in 40 ml of Me_2SO was added dropwise over 30 min to a stirred solution of the biotin and NHS. After 16 h, the *N,N'*-dicyclohexylurea which had precipitated was removed by filtration. Me_2SO was removed from the filtrate at 70°C under reduced pressure, and the resulting product purified by two successive precipitations from 1% acetic acid in DMF with ether. It was obtained in a 60% yield (3.35 g), mp $208\text{--}210^\circ\text{C}$, (lit. $208\text{--}210^\circ\text{C}$) (9).

3-(4-(N-Biotinoyl-6-aminocaproxyloxy)phenyl)propionic acid (VI). A solution composed of 8.6 mmol (1.2 ml) triethylamine in 20 ml of Me_2SO was added (during 5 min) to a stirred solution of V (1 g, 2.93 mmol) and IV (1.14 g, 3.19 mmol) in 50 ml of Me_2SO . The reaction mixture was stirred for an additional 13 min, whereupon it was poured onto 600 g crushed ice which had been acidified with 4 ml of concentrated HCl. The resulting precipitate was rinsed with 0.01 N HCl, ice water, and 20 ml of 1% acetic acid in acetonitrile; and then dried under reduced pressure at room temperature to yield 1.13 g (76%) of VI mp $199\text{--}200^\circ\text{C}$. The biotin content and phenyl ester content (see below) of this material were 99 and 97%, respectively, of that expected for the pure compound.

3-(4-(N-Biotinoyl-6-aminocaproxyloxy)phenyl)propionic acid N-hydroxysuccinimide ester (VII). This compound was prepared from VI in a manner similar to that used to prepare the *N*-hydroxysuccinimide ester of biotin. The product (mp $177\text{--}179^\circ\text{C}$) was obtained in 62% yield (732 mg) from 1 g (1.97 mmol) of VI. It exhibited phenyl ester, biotin, and *N*-hydroxysuccinimide ester contents (see below) which were 101, 99, and 100% of that expected for the pure VII. The product also chromatographed as a single component on HPLC on a 250 by 4.6 (i.d.)-mm Whatman Partisil-10 ODS C-18 column using 1% acetic acid-2% DMF-97% acetonitrile (v/v/v) as eluent. Initial preparations of VII (prepared from impure VI) yielded a crude product from which pure VII could be obtained by this HPLC procedure.

NHS-CM-Sepharose. CM-Sepharose CL-6B (20 ml) obtained from Pharmacia was washed on a sintered glass funnel with 350 ml of 0.1 N HCl, 250 ml of water, and then with 60 ml of dioxane. The washed gel was activated with NHS according to a procedure similar to that of Cuatrecasas and Parikh (3). For activation, the washed gel was stirred with NHS (691 mg, 6 mmol) and *N,N'*-dicyclohexylcarbodiimide (1.24 g, 6 mmol) in 40 ml of dioxane for 70 min. After removal of the liquid phase, the gel was washed with 150 ml of dioxane, 200 ml methanol (to remove precipitated *N,N'*-dicyclohexylurea), and 100 ml dioxane. The resulting NHS-CM-Sepharose was stored under dioxane.

Avidin Sepharose. NHS-CM-Sepharose (150 μl) was

washed with ice-cold 0.1 M phosphate buffer (pH 7.0) and added to 8.16 mg (124 nmol) of avidin (from Sigma) in 0.8 ml of ice-cold 0.1 M phosphate buffer (pH 7.0). The resulting suspension was stirred for 3 h at room temperature. The gel was then washed four times with 1.0 ml of 0.1 M phosphate buffer (pH 7.0) and shaken with 1 ml of 1.0 M ethanolamine in 1.0 M phosphate (pH 7.0) to decompose unreacted NHS ester. The resulting gel was washed six times with 1.0 ml of 0.1 M phosphate buffer (pH 7.0) and the avidin-linked gel was diluted 1:100 with gel that had been activated with NHS and then inactivated with ethanolamine without addition of avidin. The resulting gel mixture, avidin Sepharose, was suspended in sufficient 0.1 M phosphate buffer (pH 7.0) to produce a suspension that contained 100 μ l of gel/ml. Before use in studies of the binding of insulin to the gel, avidin Sepharose (100 μ l) was resuspended in 1.0 ml of 1.0 mg/ml bovine serum albumin (BSA) in 0.1 M phosphate buffer (pH 7.0) to minimize nonspecific binding of insulin.

[¹²⁵I]Insulin. Insulin (porcine, from Elanco) was iodinated with carrier free Na¹²⁵I from Amersham by a modification of the method of Freychet *et al.* (4). Insulin (100 μ g, 17.4 nmol) in 0.12 ml of 0.05 M phosphate buffer (pH 7.4) was mixed with 2 mCi Na¹²⁵I (0.91 nmol in 3.7 μ l) and 10 μ l of 0.35 mM chloramine-T in 0.05 M phosphate buffer (pH 7.4). After 5 min the reaction was quenched by addition of 5 μ l of 0.70 mM sodium metabisulfite. The iodide and insulin were separated by gel exclusion chromatography on a 17 \times 0.7 (i.d.)-cm column of Sephadex G-25 Medium, which was eluted with 0.05 M sodium phosphate buffer (pH 7.4).

[¹²⁵I]Insulin derivative of VII (BPE-insulin). Insulin (1 mg, 0.17 μ mol) in 2.0 ml of a solution containing 30% acetonitrile–70% 0.2 M sodium phosphate buffer (pH 7.0) was stirred with a tracer amount (1.75×10^7 cpm) of [¹²⁵I]insulin in 50 μ l of 0.05 M phosphate buffer (pH 7.4) and 1 mg (1.7 μ mol) VII. After 5.5 h at room temperature, the reaction was quenched by addition of 20 μ l 1 M glycine. The volume of the reaction mixture was reduced to less than 1 ml under a stream of air. The precipitate which formed was removed by centrifugation and dissolved in 1 ml 0.02 M sodium phosphate buffer (pH 7.0). The resulting solution was subjected to gel exclusion chromatography on a 39 \times 1 (i.d.)-cm column of Sephadex G-25 medium and the insulin-containing fraction which appeared in the void volume was collected.

[¹²⁵I]Insulin derivative of V (B-insulin). This was prepared by a procedure similar to the one used to prepare BPE-insulin except that material which precipitated during evaporation was not separated. Instead, any precipitated material was dissolved by addition of sufficient 0.02 M sodium phosphate buffer (pH 7.0) to obtain a final volume of 2 ml.

The phenyl ester content of compounds. This was determined from the time-dependent increase of absorbance at 298 nm due the release of the phenoxide ion which accompanies solvolysis of the phenyl ester in basic ethanol. In a typical assay, 75 μ l of a 10 mM (by weight) solution of ester in ethanol or DMF was added to 3 ml of ethanol which contained 5% by volume of a solution composed of 25% tetrabutylammonium hydroxide in methanol. The absorbance (298 nm) of the solution was monitored continuously from the time of addition of the ester. The approach of the absorbance (A) to its final value (A_t) was first order, and linear plots of $\ln(A - A_t)$ vs time were extrapolated to the time of mixing to obtain the initial absorbance (A_0) of the solution. (It should be noted that the contribution of the phenyl ester chromophore to A_0 is negligible at 298 nm). The concentration of phenyl ester [PE] in the cuvette was calculated from the relationship

$$[\text{PE}] = (A_t - A_0)/2540,$$

where 2540 $\text{cm}^{-1} \text{M}^{-1}$ is the molar absorptivity of the phenoxide ion of HPPA. The standard deviation for this analysis was less than 3%.

The biotin content of compounds. This was determined by a modification of the dye displacement method of Green (5). In a typical analysis, a 10 mM solution of the biotin-containing compound in DMF was diluted to 5 μ M with 0.05 M sodium phosphate, pH 6.5. A sample (0.82 ml) of this solution and 0.18 ml of a solution containing 1.0 mg/ml avidin and 0.17 mM 2-(4-hydroxyazobenzene)benzoic acid in water were mixed to yield a final volume of 1 ml. The absorbance of this solution (at 500 nm) was determined and related to the biotin content of the sample using the linear dependence of the absorbance on the biotin content observed for samples containing known amounts of biotin. The standard deviation for this analysis was less than 3%.

The N-Hydroxysuccinimide ester content of VII. This was determined from measurements of the absorbance change ($\epsilon_{262} = 9038 \text{ cm}^{-1} \text{M}^{-1}$) which accompanied the release of NHS anion by imidazole. The absorbance (at 262 nm) of solutions composed of 50 μ l of 10.2 mM (6.17 mg/ml) VII in DMF and 3 ml of solutions containing: 70% by volume 0.3 M sodium phosphate (pH = 7.1), 30% Me₂SO, and either 0.35 M imidazole or no imidazole. The concentration of N-hydroxysuccinimide ([NHS]) released was calculated from the equation

$$[\text{NHS}] = (A_1 - A_0)/9038,$$

where 9038 $\text{cm}^{-1} \text{M}^{-1}$ is the molar absorptivity of NHS under the assay conditions, A_1 is the observed absorbance in the presence of 0.35 M imidazole and A_0 is the observed initial absorbance of VII in the ab-

sence of imidazole. The standard deviation for this analysis was less than 3%.

General operations used with avidin Sepharose.

Gels were treated with solutions at room temperature for the indicated times by shaking the mixtures on a Burrell wrist-action shaker or by stirring them on a magnetic stirrer. Both mixing procedures gave similar results. The reaction vessel was a 75 × 12 (o.d.)-mm glass test tube, which could be used as a sample container in the gamma counter (Searle Analytic Inc., Model 1190) for determinations of radioactive material in the gel phase. Gels were washed by treating them for 1 min with the specified wash solution. Solutions were separated from gels by centrifugation on an International Clinical desk top centrifuge. Prior to determination of their radioactivity aliquots of the supernatant solutions were subjected to a second centrifugation in a Beckman microfuge to ensure removal of trace amounts of gel.

Reaction of insulin derivatives with avidin Sepharose. Avidin Sepharose (0.1 ml) was suspended in 0.5 ml of 0.1 M sodium phosphate buffer, 0.1% BSA (pH 7.0) and treated for 4 h with the indicated amounts of BPE-insulin or B-insulin. The unbound insulin derivative was removed by centrifugation and the gel was washed three times with 1 ml of 0.1 M phosphate buffer, 1 mg/ml BSA (pH 7.0) and once by treating the gel for 40 min with 1 ml of a buffer consisting of 0.1 M phosphate, 1 M NaCl, 1 mg/ml BSA (pH 7.0). The radioactivity of the washes was monitored as well as the radioactivity of the washed gel. The last wash contained less than 2% of the radioactivity of that which was present in the washed gel.

Cleavage of phenyl ester linkages and release of gel-bound insulin. This was accomplished by treating 0.1 ml gel for 40 min with 1 ml of 1.0 M NH₂OH, 1 mg/ml BSA (pH 7.0). The hydroxylamine solution was prepared by addition of BSA to NH₂OH:HCl which had been neutralized with NaOH to pH 7.0, and adjusted to 1 M NH₂OH. After reaction with NH₂OH the gel was washed three times with 1 ml of 0.1 M phosphate buffer, 1 mg/ml BSA (pH 7.0). The release of radioactive insulin derivative from the gel was monitored by determination of the radioactivity in the gel and liquid phases.

The biotin binding capacity of avidin Sepharose. This was determined by treating a 1.0-ml aliquot of avidin Sepharose suspension for 1 h with 50 μl of a solution containing a known concentration of biotin in water. The amount of biotin which bound to the gel was determined from the difference between the amount of biotin added and the amount of unbound biotin in the supernatant solution obtained after removal of the gel by centrifugation. The amount of biotin in the supernatant solution was determined in a manner similar to that described for the determination of the biotin content of organic compounds.

Biotin standards in the same buffer as the gel supernatant solution were used to calibrate the assay.

RESULTS AND DISCUSSION

Reaction of BPE with insulin resulted in covalent attachment of biotin to insulin as evidenced by the behavior of the resultant BPE-insulin toward avidin Sepharose (see below). The reaction of BPE with insulin should be analogous to that reported for the reaction of biotin *N*-hydroxysuccinimide ester (V) with insulin wherein *N*-hydroxysuccinimide is displaced from V by the amino groups of insulin with concomitant formation of amide bonds (9). The biotin in the insulin derivative of V (B-insulin), however, is linked to the insulin via a noncleavable linkage, whereas in BPE-insulin the biotin is attached to the insulin via a cleavable connector arm. Since there are three amino groups in insulin, seven derivatives can result from the reaction of insulin with BPE or V.⁵ In the procedure used in this work to label insulin with BPE or V, a greater than ninefold molar excess of BPE or V was reacted with insulin for 5.5 h to ensure that essentially all of the insulin molecules would be covalently linked to at least one biotin group.⁶ Furthermore, the insulin was reacted in the presence of tracer amounts of [¹²⁵I]insulin to facilitate quantification of the binding of the resulting BPE-insulin or B-insulin to immobilized avidin (avidin-Sepharose). The titrations

⁵ The seven insulin derivatives of V and BPE have been separated by HPLC. The preparation and physiological properties of these derivatives will be described elsewhere.

⁶ The derivitized insulins used here are primarily trisubstituted with biotin. To obtain the other BPE-insulin derivatives, a mixture of the seven derivatives was prepared by reaction of a ninefold molar excess of BPE with 2 mg/ml insulin in a solution composed of equal volumes of acetonitrile and 0.2 M sodium phosphate buffer (pH 6.6 before mixing). After 45 min the reaction mixture was quenched by addition of 0.1 volume of 2 M phosphoric acid. The seven BPE-insulin derivatives were isolated from each other and unreacted insulin by HPLC on a C-8 column using an acetonitrile-sodium phosphate (pH 3.1) gradient as eluent.

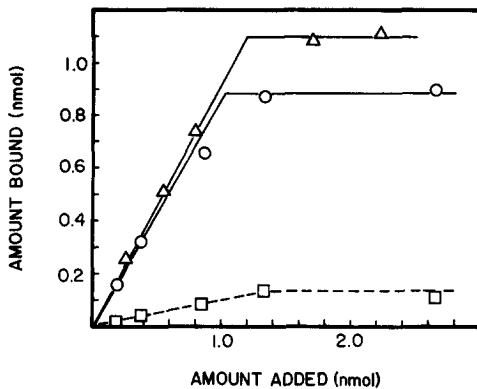


FIG. 1. Titration of avidin Sepharose with BPE-insulin and B-insulin. The indicated amounts of BPE-insulin (○) and B-insulin (Δ) were stirred for 4 h with 0.1 ml of avidin Sepharose in 0.1 M sodium phosphate buffer (pH 7) in a total volume of 1.03–1.3 ml. The squares indicate the amount of BPE-insulin remaining bound after treatment of the bound insulin (○) with 1 M hydroxylamine (pH 7) for 40 min at room temperature. Treatment of bound B-insulin (Δ) with hydroxylamine resulted in no change (<2%) in the amount of B-insulin bound to the avidin Sepharose.

of avidin Sepharose depicted in Fig. 1 yield binding capacities of 11 nmol B-insulin and 8.9 nmol BPE-insulin per milliliter of avidin Sepharose. Separate determinations of the capacity of the gel to bind free biotin indicated that the avidin Sepharose could bind 31 nmol of biotin per milliliter of gel. The approximately three-fold lower binding capacity observed with BPE-insulin and B-insulin may well reflect, in part, the ability of the polyvalent biotinylated insulin derivatives to fill more than one biotin binding site. Limited access of the insulin derivatives to the biotin binding sites of the immobilized avidin could also account for the lower capacity of the gel to bind insulin. It should be noted that the avidin Sepharose used in this work resulted from a 100-fold dilution of immobilized avidin with inert gel (see under Materials and Methods). Thus, gels having as much as a 100-fold higher capacity to bind biotin can be obtained simply by diluting the immobilized avidin less.

The initial slopes of 0.93 and 0.85 observed for the plots of the amount of B-insulin and BPE-insulin bound versus the

amount of insulin derivative added (Fig. 1) indicate that essentially all of the derivatized insulin was competent to bind to the avidin Sepharose. In the presence of excess avidin Sepharose, binding of biotinylated insulin proceeded at a reasonable rate. For example, 0.17 nmol of BPE-insulin bound to 0.1 ml of avidin Sepharose (containing 0.89 nmol of sites for BPE-insulin) with a half-time of 8 min. This half-time for binding could be decreased by using gels which contained higher concentrations of immobilized avidin. As the amount of biotinylated insulin approached the capacity of the avidin Sepharose, the time required to ensure complete reaction (e.g., in the titrations depicted in Fig. 1) increased from 1 to 4 h. The decreased rate of binding with increased degree of saturation may well reflect the existence of a population of sterically hindered biotin binding sites which reacts more slowly with biotinylated insulin.

Previous studies from this laboratory (12, 13) demonstrated that a phenyl ester linkage such as the one in BPE-insulin is readily cleaved at 25°C by treatment with 1 M hydroxylamine at pH 7.0. Figure 1 illustrates the greater than 85% release of BPE-insulin from avidin Sepharose that we obtained upon treatment of the bound BPE-insulin with 1 M hydroxylamine (pH 7.0) for 40 min at room temperature. However, well over 90% of the releasable BPE-insulin was released within 20 min. The failure to observe quantitative release of the BPE-insulin from the avidin Sepharose appears to be due to nonspecific interactions between the BPE-insulin and the Sepharose. Thus, the inert Sepharose derivative that was used to dilute the immobilized avidin (see under Materials and Methods) exhibited a binding capacity (after incubation of 0.1 ml gel with 2.7 nmol BPE-insulin) which was 1.2 nmol/ml gel. Most of this nonspecifically bound insulin (0.9 nmol/ml gel) was not released by treatment with hydroxylamine. This amount of nonreleasable BPE-insulin accounts for most of the nonreleasable BPE-insulin (1.1 nmol/ml gel) which was observed when 0.1 ml avidin Sepharose was

incubated with 2.7 nmol of BPE-insulin and then treated with hydroxylamine (Fig. 1). It should be noted that the nonspecific binding of proteins to the inert gel diluent can be minimized by the use of smaller volumes of gels which contain a higher fraction of the immobilized avidin.

Several control experiments were performed to verify that binding of BPE-insulin to the avidin Sepharose was dependent on the biotin:avidin interaction and that the subsequent release of bound insulin from the gel was due to hydroxylamine-mediated cleavage of the phenyl ester-containing connector arm. Little binding (<5% of that expected for BPE-insulin) was observed when avidin Sepharose was incubated with excess [¹²⁵I]-insulin which had not been treated with BPE. Avidin Sepharose that had been treated with hydroxylamine and then washed with buffer was indistinguishable from untreated avidin Sepharose with respect to its ability to bind biotin or BPE-insulin. Incubation of the avidin Sepharose with biotin prior to treatment of the gel with BPE-insulin resulted in binding which was at the level seen for the nonspecific binding of BPE-insulin to the inert Sepharose diluent. Similarly, when avidin Sepharose was saturated with BPE-insulin and then treated with hydroxylamine to release the bound insulin from the gel, the resulting avidin Sepharose had a capacity for additional BPE-insulin which was less than 10% of that observed for avidin Sepharose which had not been preincubated with BPE-insulin and hydroxylamine. These results suggest that the pretreatment of the avidin Sepharose with BPE-insulin and hydroxylamine, like the pretreatment of avidin Sepharose with free biotin, saturated the insulin-accessible biotin binding sites on the immobilized avidin, and thereby prevented subsequent binding of BPE-insulin.

In the absence of added hydroxylamine, bound BPE-insulin was not released from avidin Sepharose by extensive washing with neutral buffers at either low or high concentrations of salt. The view that the release of insulin from avidin-bound BPE-insulin is dependent upon hydroxylamine-

mediated cleavage of the phenyl ester linkage was further substantiated by studies with B-insulin wherein the biotin label is connected to insulin via a connector arm which should not be cleaved by the hydroxylamine treatment. As shown in Fig. 1, avidin Sepharose bound B-insulin and BPE-insulin similarly. However, less than 2% of the bound B-insulin was released from the avidin Sepharose by the hydroxylamine treatment.

The possibility that the hydroxylamine treatment described in this work could result in chemical modification of protein should be considered. Bornstein and Balbian (2) have shown that treatment of reduced carboxymethylated ribonuclease for 2 h at 45°C with 2 M hydroxylamine in 0.2 M K₂CO₃ at pH 9 resulted only in cleavage at an Asn-Gly bond and 5-10% cleavage at an Asn-Leu bond. It should be noted that this hydroxylamine-mediated cleavage requires hydroxide ion-catalyzed attack of the nitrogen atom of the amide backbone on the β-carboxamido group of the Asn to displace ammonia and form a cyclic imide. It is this imide intermediate which is then cleaved upon attack by the nucleophile hydroxylamine (1). Hydroxide ion-catalyzed formation of the imide intermediate should be several hundred times slower at pH 7.0, 25°C than at pH 9.0, 45°C. Thus, hydroxylamine-mediated peptide bond cleavage should not be significant under the conditions used to cleave the phenyl ester linkage. Naturally, one cannot *a priori* exclude the possibility that some proteins might contain a bond which is reactive toward hydroxylamine. Should this become a concern, however, one could use a weaker nucleophile such as imidazole to cleave the phenyl ester linkage (12).

The selective binding of BPE-insulin to avidin and its subsequent release suggests that BPE will enable us to exploit fully the high affinity of avidin for biotin in the design of protocols for selective retrieval of biotinylated compounds from cells and physiological fluids. In the case of insulin, it is likely that some of the BPE-insulin derivatives will be useful for the isolation and characterization of the insulin receptor, since biotinylated insulin derivatives

are competent to bind the insulin receptor of rat epididymal fat cells and induce insulin-like effects on metabolism (9), and unpublished observations of this laboratory). A possible limitation of the use of the biotin label (as well as any other probe) is that the label itself might modify the physiological properties of the polypeptide so as to produce "artifactual" behavior. Determination of the behavior of several different polypeptide derivatives should minimize this problem, however.

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