Avidin Binding of Carboxyl-Substituted Biotin and Analogues†

Klaus Hofmann,* Gail Titus, Judith A. Montibeller, and Frances M. Finn

ABSTRACT: The use of biotinylated hormones as ligands on avidin–Sepharose columns represents an attractive approach to the isolation of receptors. However, the strong noncovalent interaction between the hormone and its receptor on one hand and biotin and avidin on the other poses major obstacles to receptor retrieval. We report the results of a systematic search for weaker binding biotin containing ligands that are displaceable by biotin. Syntheses are described of the *N*-hydroxysuccinimido esters of iminobiotin, dethiobiotin, 6-(iminobiotinylamido)hexanoic acid, and 6-(dethiobiotinylamido)hexanoic acid. These active esters served to acylate $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin (from porcine insulin), and the resulting protected insulins were converted to N^{α,B^1}-iminobiotinyl-, N^{α,B^1}-dethiobiotinyl-, N^{α,B^1}-[6-(iminobiotinylamido)hexyl]-, and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]-insulins, respectively. Complexes of these insulin derivatives with avidin and succinoylavidin were prepared, and the rate of their dissociation (off-reaction) was determined in the presence of [^{14}C]biotin. It was found that the attachment of the insulin molecule to biotin and the biotin analogues exerted a striking effect on the rate of dissociation of the complexes. The half-time for dissociation of the avidin–biotin complex is 200 days and that of the succinoylavidin–biotin complex is 127 days. The complex of biotinylinsulin with avidin or succinoylavidin is 50% dissociated in approximately 1 h. The half-time for dissociation of the dethiobiotin–succinoylavidin complex is 11 h, and in contrast the dethiobiotinylinsulin–succinoylavidin complex is 88% dissociated immediately after mixing with [^{14}C]biotin. At pH 9.0 iminobiotinylinsulin fails to form a complex with either avidin or succinoylavidin. The interposition of a spacer (6-aminohexanoic acid) between the biotin derivatives and insulin exerts a stabilizing effect on their complexes with avidin or succinoylavidin. This is observed with both [6-(iminobiotinylamido)hexyl]- and [6-(dethiobiotinylamido)hexyl]insulins. N^{α,B^1}-Biotinyl-, N^{α,B^1}-dethiobiotinyl-, and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulins were labeled with ^{125}I, and the labeled compounds were noncovalently attached to AH-Sepharose 4B immobilized succinoylavidin. Exposure of the ensuing resins to buffer containing 20 mM biotin resulted in displacement of the ligand. Under the most favorable conditions, approximately 50–60% of the ligand could be retrieved. These experiments indicate that the attachment of insulin to biotin or biotin analogues imposes a steric impediment to the interaction of the biotin residues with their avidin or succinoylavidin binding sites. The observation that insertion of a spacer between the avidin binding site and the insulin molecule increases the stability of the complexes supports this view.

In recent publications (Hofmann & Kiso, 1976; Hofmann et al., 1977, 1978) we have proposed an approach to the isolation of polypeptide and protein hormone receptors that is based on the strong noncovalent interaction of biotin with avidin. To this end we have synthesized [25-biocytin]-$ACTH_{1-25}$ amide (Hofmann et al., 1978) and N^{α,B^1}-biotinylinsulin (Hofmann et al., 1977) and have determined that these modified hormones exhibit the same biological activity as their natural counterparts (Hofmann & Kiso, 1976; Hofmann et al., 1977). We have also attached avidin to the biotinylated hormones and have observed that the ensuing complexes retained the ability to bind to their specific receptors on the target cells and exhibited biological activity (Hofmann et al., 1977; Finn et al., 1979–1981). Further studies (Finn et al., 1980) with radioiodinated avidin showed that this molecule binds unsaturably to rat liver plasma membranes. Exhaustive succinoylation of avidin markedly reduced its affinity for membranes without significantly altering its biotin binding properties. On the basis of the results of these studies, we have concluded that the attachment of avidin or succinoylavidin to defined sites on the hormone molecules does not prevent binding to their specific receptors on cells and plasma membranes.

The very strong noncovalent association between avidin and biotinylated hormones poses a major obstacle to the proposed receptor retrieval and to isolation schemes that are based on the avidin biotin interaction. We reasoned that the availability of biotin derivatives having lower binding affinity for avidin than biotin could facilitate retrieval of biotinylhormone–receptor complexes from avidin or succinoylavidin columns. We have synthesized N^{α,B^1}-iminobiotinylinsulin, N^{α,B^1}-dethiobiotinylinsulin, N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin (Figure 1) and have investigated their interaction with avidin and succinoylavidin in solution and also their binding to and retrieval from Sepharose 4B immobilized succinoylavidin columns. The results of these studies are described in this paper.

Materials and Methods

Avidin was from Sigma. Succinoylavidin was prepared as described (Finn et al., 1980). Biotin was a gift from Dr. W. E. Scott of Hoffmann-LaRoche Inc., Nutley, NJ, and [^{14}C]biotin (specific activity 48 mCi/mmol) was from Amersham. Sephadex G-50, Sephadex G-25, and AH-Sepharose 4B were from Pharmacia. Diethylaminoethylcellulose DE52 was from Whatman Ltd., Springfield Hill, Maidstone, Kent, England, and Amberlite ion-exchange resin IRA-400 was from Mallinckrodt Chemical Works. CMC Cellex-CM was from Bio-Rad Laboratories. $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin[1] (porcine) was a gift from Professor R. Geiger and Dr. R. Obermeier of Höchst A.G., Frankfurt am Main, West Germany. 5-(3,4-

† From the Protein Research Laboratory and the Department of Medicine, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261. *Received September 11, 1981.* Supported by National Institutes of Health Grants AM 01128 and AM 21292. Presented in part at the 7th American Peptide Symposium in Madison, WI, June 14–19, 1981.

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; Me_2SO, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography; TEA, triethylamine; TLC, thin-layer chromatography; Z, benzyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; DMF, dimethylformamide.

0006-2960/82/0421-0978$01.25/0 © 1982 American Chemical Society

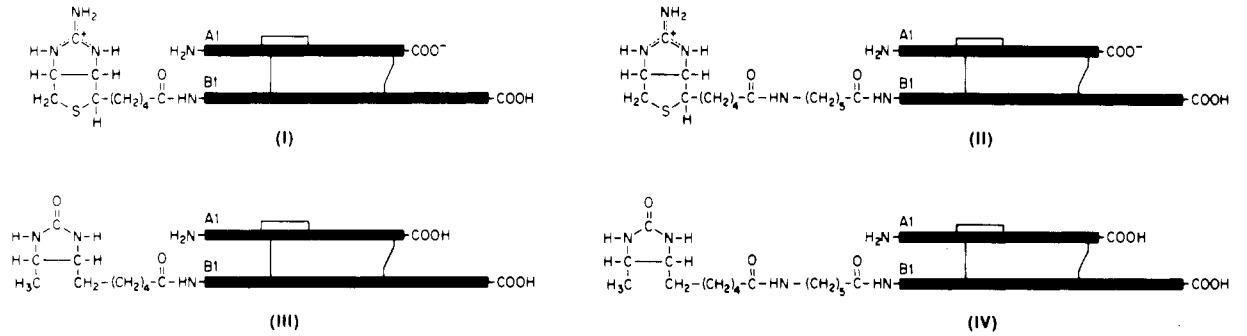


FIGURE 1: Simplified structures of N^{α,B^1}-iminobiotinylinsulin (I), N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin (II), N^{α,B^1}-dethiobiotinylinsulin (III), and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin (IV).

Diaminothiophan-2-yl)pentanoic acid sulfate was a gift from Dr. R. Andreatta of CIBA-Geigy Corp., Basel, Switzerland. N^{α,B^1}-Biotinylinsulin was prepared as described (Hofmann et al., 1977). *N*-Hydroxysuccinimido trifluoroacetate was prepared according to Sakakibara & Inukai (1965) who described the compound as an oil. We have obtained this material in the form of deliquescent, hydroscopic crystals. For the binding studies, the various insulin derivatives were labeled with ^{125}I as described previously (Finn et al., 1980), and the labeled materials were purified on Sephadex G-50. The iminobiotinyl derivatives were also labeled with ^{14}C by the procedure of Rice & Means (1971). The solvent systems for ascending TLC on silica gel (E. Merck and Co., Darmstadt, West Germany) were R_f^I 1-butanol–glacial acetic acid–water (60:20:20), R_f^{II} chloroform–methanol–water (8:3:1, upper phase), and R_f^{III} 1-butanol–pyridine–glacial acetic acid–water (30:20:6:24). The biotin derivatives were visualized with the chlorine reagent. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, NJ. High-pressure liquid chromatography was performed with a Waters HPLC system equipped with a Model 660 solvent programmer.

AH-Sepharose 4B Immobilized Succinoylavidin. AH-Sepharose 4B (1 g) was swollen in 0.5 M NaCl (200 mL), and the resin was washed with 0.5 M NaCl (200 mL) and water (200 mL). The resin was slurried with water to a volume of 10 mL, succinoylavidin (10 mg) was added, and the pH was adjusted to 9.0 with 1 N NaOH. The slurry was stirred for 30 min, the pH was adjusted to 4.8 with 1% HBr, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (423 mg) was added slowly with stirring. The pH was kept at 4.8 by addition of 1% HBr. After 3 h there was little change in pH, and stirring was continued for 12 h. The resin was collected, washed with 0.5 M NaCl (200 mL) and water (200 mL), and stored as a 50% slurry in water containing 0.1% of NaN_3. The biotin binding capacity of several batches ranged from 80 to 100 nmol of biotin/mL of settled resin.

Ligand Binding and Displacement Studies. (*a*) *In Solution.* Complexes were prepared by mixing succinoylavidin (1 equiv) in 50 mM Tris-HCl, pH 7.6, with desired ligand (8 equiv; 2-fold excess) in 0.01 N HCl (4 mg/mL), and excess ligand was removed by gel filtration on Sephadex G-50 using 50 mM Tris-HCl, pH 7.6, as the solvent. The concentration of the complex in the high molecular eluates was determined spectrophotometrically, and a 100-fold excess of [^{14}C]biotin was added. Suitable aliquots of this solution were subjected to gel filtration on Sephadex G-50 immediately after mixing or following incubation at room temperature for specified times. The radioactivity in the protein eluates provided a measure of the rate of dissociation.

(*b*) *With Immobilized Succinoylavidin.* AH-Sepharose 4B immobilized succinoylavidin (0.35 mL of settled resin) was placed in a Pasteur pipet and washed with 50 mM Hepes, 0.5 M NaCl, and 0.1% Triton X-100, pH 7.6, buffer (HST). The desired amount of ^{125}I-labeled Sephadex G-50 purified ligand [N^{α,B^1}-biotinylinsulin, N^{α,B^1}-dethiobiotinylinsulin, or N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin in HST was added, and the pipet was closed and rotated for 30 min at room temperature. The column was then washed with HST (250 mL) at 4 °C, and the radioactivity in the column flow-through and washings was determined. The washed column was then eluted by rotation for 3 h at room temperature in HST containing 20 mM biotin. Radioactivity in the eluate was determined, and the concentration of the ligand was calculated from the specific activity of the applied sample.

Synthetic Aspects. Iminobiotin. 5-(3,4-Diaminothiophan-2-yl)pentanoic acid sulfate (Hofmann et al., 1941) (1.26 g, 4.0 mmol) was dissolved in 0.5 N NaOH (16 mL, 8 mmol), and a solution of cyanogen bromide (2.183 g in acetonitrile, 5 mL) (1.63 mL, 6.7 mmol) was added at room temperature with stirring. A heavy precipitate soon formed, and the suspension was stirred for several hours while 1 N NaOH was added from time to time to maintain the pH at 7.0–7.5 (Hydrion paper). The precipitate was collected, washed with water, and dried at 100 °C in vacuo: yield 886 mg (92%); mp above 200 °C dec; Hofmann & Axelrod, 1950) mp above 260 °C dec; $[\alpha]^{25}_D$ +79.7° (*c* 1.053, N HCl); R_f^I 0.47. The product was halogen free. Anal. Calcd for $C_{10}H_{17}N_3O_2S$: C, 49.36; H, 7.04; N, 17.27. Found: C, 49.22; H, 7.28; N, 17.05.

N-Hydroxysuccinimido Iminobiotinate Hydrobromide. Iminobiotin hydrobromide prepared from iminobiotin and aqueous HBr was dried over P_2O_5 and KOH pellets. The hydrobromide (667 mg, 2.06 mmol) and *N*-hydroxysuccinimide (260 mg, 2.26 mmol) were dissolved in freshly distilled DMF (6 mL), and the solution was cooled in an ice bath. DCC (466 mg, 2.26 mmol) dissolved in a small amount of DMF was added, and the solution was stirred at ice-bath temperature for 1 h and at room temperature for 20 h. The suspension was cooled in an ice bath, the DCU was removed by filtration, and the filtrate was evaporated to dryness. The residue was washed with two portions of peroxide-free ether and dried. For crystallization the compound was dissolved in boiling 2-propanol (approximately 75 mL), and the clear solution was evaporated to a volume of approximately 25 mL and kept at room temperature for 20 h. The crystals were collected, washed with ice-cold 2-propanol and dried; yield 550 mg (63%); mp 176–178 °C [lit. (Orr, 1981) mp 160–161 °C]; $[\alpha]^{25}_D$ +48.5° (*c* 1.28, H_2O); R_f^I 0.4; R_f^{III} 0.5. Anal. Calcd for $C_{14}H_{21}N_4O_4SBr$: C, 39.91; H, 5.02; N, 13.30; S, 7.61. Found: C, 40.25; H, 5.20; N, 13.26; S, 7.61.

N-Hydroxysuccinimido Dethiobiotinate. Dethiobiotin (Melville et al., 1943) (428 mg, 2 mmol) and *N*-hydroxysuccinimide (254 mg, 2.2 mmol) were dissolved in DMF (2

mL), and DCC (412 mg, 2 mmol) dissolved in a small volume of DMF was added. The solution was stirred for 10 h at room temperature and was then placed in a refrigerator for 2 h. The DCU was removed by filtration, and the filtrate was evaporated to dryness in vacuo. The residue was dissolved in 2-propanol, insoluble yellow material was removed by filtration, and the filtrate was concentrated to a small volume. The solution was placed in a refrigerator where crystallization occurred. The crystals were collected, washed with ice-cold 2-propanol, and dried: yield 261 mg (42%). A sample for analysis was recrystallized from 2-propanol–ether: mp 144–145 °C; $[\alpha]^{27}_D$ −1.2° (*c* 1.241, DMF); R_f^I 0.5; R_f^{II} 0.8; R_f^{III} 0.6. Anal. Calcd for $C_{14}H_{21}N_3O_5$: C, 54.01; H, 6.80; N, 13.50. Found: C, 53.89; H, 6.73; N, 13.34.

Iminobiotinyl-L-phenylalanine Monohydrate. A 10% solution of TEA in DMF (0.69 mL, 0.5 mmol) was added to a solution of L-Phe-OMe·HCl (108 mg, 0.5 mmol) and *N*-hydroxysuccinimido iminobiotinate hydrobromide (232 mg, 0.55 mmol) in DMF (4 mL), and the solution was stirred at room temperature for 12 h. The reaction mixture was evaporated to dryness, and 1 N NaOH (2 mL) was added to the oily residue followed by sufficient MeOH to give a clear solution. The solution was stirred at room temperature for 1 h during which time a heavy precipitate had formed. The flask was evacuated for a short time to remove the TEA, and 1 N HCl (1.5 mL) was added to the residue. The precipitate was dissolved by warming, and the pH was adjusted to 7.5 with 1 N NaOH (Hydrion paper). The solution was placed in a refrigerator for 12 h, and the crystals were collected and recrystallized from boiling water to give rosettes: yield 126 mg (52%); mp 181–182 °C; $[\alpha]^{28}_D$ +69.4° (*c* 0.523, hydrochloride in EtOH); R_f^I 0.4; R_f^{III} 0.5; acid hydrolysis gave Phe. Anal. Calcd for $C_{19}H_{26}N_4O_3S·H_2O$: C, 55.86; H, 6.91; N, 13.72. Found: C, 55.51; H, 6.78; N, 13.37.

Iminobiotinylglycine Semihydrate. This compound was prepared from Gly–OMe·HCl (63 mg, 0.5 mmol) and *N*-hydroxysuccinimido iminobiotinate hydrobromide (231 mg, 0.55 mmol) in DMF (4 mL) and TEA (1:10 in DMF) (0.69 mL, 0.5 mmol) as described for the preparation of the Phe analogue. The compound was recrystallized from boiling water: yield 109 mg (70%); mp above 250 °C; $[\alpha]^{25}_D$ +65.7° (*c* 1.111, 1 N HCl); R_f^I 0.23; R_f^{III} 0.36. Anal. Calcd for $C_{12}H_{20}N_4O_3S·0.5H_2O$: C, 46.59; H, 6.84; N, 18.11. Found: C, 46.66; H, 6.73; N, 17.88.

N^ϵ-Iminobiotinyl-L-lysine Amide Diacetate.[2] N^α-Boc-N^ϵ-Z-L-lysine amide (379 mg, 1 mmol) was hydrogenated over palladium as described previously (Hofmann et al., 1978), and the reaction product was dried over P_2O_5 and KOH pellets. To a solution of the hydrogenation product in DMF (6 mL) was added *N*-hydroxysuccinimido iminobiotinate hydrobromide (463 mg, 1.1 mmol) followed by TEA (0.14 mL, 1 mmol), and the solution was stirred at room temperature for 24 h. The solvent was removed, and the residue was triturated with ethyl acetate and dried. The residue was dissolved in 90% F_3CCOOH, and the solution was kept at room temperature for 35 min. The bulk of the TFA was removed in vacuo, and the product was precipitated with ether, washed with ether, and dried. This material was dissolved in 10% acetic acid, and trifluoroacetate ions were exchanged for acetate ions on a column of Amberlite IRA-400 (1.8 × 14 cm). Fractions containing the desired material were concentrated to a small volume in vacuo and lyophilized. The residue was dissolved in water (100 mL), and 50 mL of the solution was applied to a column of CMC (2.2 × 14 cm) which was eluted with 350 mL of water and 2000 mL of 0.03% acetic acid. Acetic acid fractions containing the desired material (chlorine test on TLC) were pooled, evaporated to a small volume, lyophilized, and dried: yield (2 runs) 392 mg (85%); $[\alpha]^{26}_D$ +45.0° (*c* 0.96, 5% acetic acid); R_f^I 0.1; R_f^{III} 0.4. Anal. Calcd for $C_{16}H_{32}N_6O_2S·2CH_3COOH$: C, 49.2; H, 7.8; N, 17.2. Found: C, 48.6; H, 7.7; N, 17.2.

6-(Iminobiotinylamido)hexanoic Acid Dihydrate. This compound was prepared from methyl 6-aminohexanoate hydrochloride (182 mg, 1.0 mmol) and *N*-hydroxysuccinimido iminobiotinate hydrobromide (463 mg, 1.1 mmol) in DMF (4 mL) and TEA (1:10 in DMF) (1.38 mL, 1 mmol) as described for the preparation of the Phe analogue: yield 322 mg (82%). For analysis the compound was recrystallized from 20 mL of boiling water: mp above 250 °C; $[\alpha]^{27}_D$ +52.3° (*c* 1.257, 1 N HCl); R_f^I 0.3; R_f^{III} 0.5. Anal. Calcd for $C_{16}H_{28}N_4O_3S·2H_2O$: C, 48.96; H, 8.22; N, 14.27. Found: C, 49.45; H, 8.02; N, 14.39.

6-(Dethiobiotinylamido)hexanoic Acid. Triethylamine (0.22 mL, 1.59 mmol) was added with stirring to a solution of *N*-hydroxysuccinimido dethiobiotinate (495 mg, 1.59 mmol) and methyl 6-aminohexanoate hydrochloride (289 mg, 1.59 mmol) in DMF (6 mL), and the solution was stirred at room temperature for 17 h. The solvent was evaporated in vacuo, and ethyl acetate (25 mL) was added to the residue. The suspension was washed with 1 N HCl (10 mL), 1 N $NaHCO_3$ (10 mL), and saturated NaCl (10 mL). The aqueous layers were back-extracted with five 25-mL portions of ethyl acetate. The pooled ethyl acetate solutions were dried and evaporated: yield 483 mg. This material was suspended in 1 N NaOH (4 mL), and enough methanol was added to give a clear solution which was stirred at room temperature for 1 h. The solution was acidified to Congo red with 1 N HCl, and the ensuing crystal suspension was kept in a refrigerator for 1 h. The crystals were collected, washed with a small volume of ice-cold water, and dried: yield 408 mg (79%). A sample for analysis was recrystallized from boiling water: mp 161–162 °C; $[\alpha]^{27}_D$ +10° (*c* 1.105, 0.1 N NaOH); R_f^I 0.6; R_f^{II} 0.2; R_f^{III} 0.7. Anal. Calcd for $C_{16}H_{29}N_3O_4$: C, 58.69; H, 8.93; N, 12.83. Found: C, 58.67; H, 8.99; N, 12.68.

N-Hydroxysuccinimido 6-(Iminobiotinylamido)hexanoate Hydrochloride. 6-(Iminobiotinylamido)hexanoic acid dihydrate (169 mg, 0.43 mmol) was suspended in water, and 1 N HCl (0.43 mL, 0.43 mmol) was added. The solution was evaporated to dryness, and the residue was dried for 12 h in vacuo over P_2O_5 and KOH pellets. To a solution of this hydrochloride and *N*-hydroxysuccinimide (50 mg, 0.43 mmol) in DMF (2 mL) was added DCC (90 mg, 0.43 mmol) in DMF, and the mixture was stirred at room temperature for 12 h. The DCU was removed by filtration, and the filtrate was evaporated. The residue was washed with two portions of ether and dissolved in a small volume of 2-propanol, and the solution was kept in a refrigerator for 2 h when the small precipitate (DCU) was removed by filtration. The 2-propanol was evaporated and the residue triturated and washed with ethyl acetate and dried in vacuo: yield 149 mg of crude material.

N-Hydroxysuccinimido 6-(Dethiobiotinylamido)hexanoate. *N*-Hydroxysuccinimido trifluoroacetate (272 mg, 1.29 mmol) was added to an ice-cold solution of 6-(dethiobiotinylamido)hexanoic acid (264 mg, 0.81 mmol) in dry pyridine (4 mL), and the mixture was stirred at room temperature for 1 h. The pyridine was removed in vacuo, and the resulting oil was triturated with several portions of ether to give a dry

[2] This experiment was performed by Dr. H. Romovacek.

powder which was recrystallized from 2-propanol–ether: yield 302 mg (88%); mp 103–105 °C; $[\alpha]^{28}_D$ −3.0° (*c* 0.934, DMF); R_f^{II} 0.65. Anal. Calcd for $C_{20}H_{32}N_4O_6$: C, 56.59; H, 7.60; N, 13.20. Found: C, 56.31; H, 7.60; N, 13.08.

N^{α,B^1}-Iminobiotinylinsulin. A solution of $N^{\alpha,A^1},N^{\epsilon,B^1}$-$Boc_2$-insulin (porcine) (500 mg), imidazole (100 mg), and *N*-hydroxysuccinimido iminobiotinate hydrobromide (100 mg) in Me_2SO (15 mL) was stirred at room temperature for 6 h. The reaction mixture was cooled in an ice bath, and ice–water (30 mL) was added. A precipitate formed which was redissolved by addition of a small volume of Me_2SO. The solution was desalted on a column of Sephadex G-25 (6 × 115 cm) by using 0.05 M ammonium bicarbonate as the solvent. Fractions (5 mL each) were collected, and those containing the protein peak were pooled and lyophilized. The product was dried in vacuo over P_2O_5 and KOH pellets. The dried protein was dissolved in anhydrous F_3CCOOH (15 mL), the mixture was kept at room temperature for 30 min, and the bulk of the F_3CCOOH was removed in vacuo. Ether was added to the residue, and after standing in a freezer for 2 h the precipitate was collected, washed with ether, and dried (yield 456 mg). The deprotected protein was purified on a column of DE-52 (2.6 × 55 cm) as described previously (Hofmann et al., 1977). Fractions containing the major protein peak were pooled, desalted (in two batches) on a column of Sephadex G-25 (4 × 35 cm) by using 0.05 M ammonium bicarbonate as the eluant, and lyophilized. On HPLC the compound eluted at a different position from porcine insulin (Finn & Hofmann, 1981). An acid hydrolysate of the dansylated compound (Gray, 1972) did not contain dansyl-Phe.

N^{α,B^1}-[6-(Iminobiotinylamido)hexyl]insulin. *N*-Methylmorpholine (20 μL, 200 μmol) was added to a solution of $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin (porcine) (31 mg, 5 μmol) and crude *N*-hydroxysuccinimido 6-(iminobiotinylamido)hexanoate (20 mg) in Me_2SO (2 mL), and the solution was stirred for 40 h at room temperature. The reaction mixture was poured into 100 mL of ice-cold ether, and the suspension was kept in a freezer for 1 h. The precipitate was collected, washed with ether, dried, and desalted on a Sephadex G-25 column (0.9 × 55 cm). Protein containing fractions were lyophilized, and the residue was dried over P_2O_5 and KOH pellets in vacuo. This material was dissolved in anhydrous F_3CCOOH (0.5 mL), the solution was kept at room temperature for 30 min, and the product was precipitated with ether, collected, and dried. Trifluoroacetate ions were exchanged for acetate ions on a column (0.9 × 6 cm) of acetate cycle IRA 400 (20–50 mesh), and the product was lyophilized: yield 30 mg. On HPLC the compound eluted at a different position from porcine insulin (Finn & Hofmann, 1981), and the ratio of 6-aminohexanoic acid/lysine in an acid hydrolysate was 1.2; 6-aminohexanoic acid elutes from the small column of the amino acid analyzer in front of lysine (20 min).

N^{α,B^1}-Dethiobiotinylinsulin. This compound was prepared from $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin (porcine) (31 mg, 5 μmol), *N*-hydroxysuccinimido dethiobiotinate (13 mg, 40 μmol), and *N*-methylmorpholine (20 μL, 200 μmol) in Me_2SO (2 mL) as described for the preparation of N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin: yield 31 mg. On HPLC the compound eluted at a different position from porcine insulin (Figure 2), and an acid hydrolysate of the dansylated compound did not contain dansyl-Phe.

N^{α,B^1}-[6-(Dethiobiotinylamido)hexyl]insulin. This compound was prepared from $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin (porcine) (31 mg, 5 μmol), *N*-hydroxysuccinimido 6-(dethiobiotinylamido)hexanoate (17 mg, 40 μmol), and *N*-methylmorpholine (20 μL, 200 μmol) in Me_2SO (2 mL) as described for the preparation of N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin: yield 31 mg. On HPLC the compound eluted at a different position from porcine insulin (Figure 2), and the ratio of 6-aminohexanoic acid/lysine in an acid hydrolysate was 0.97.

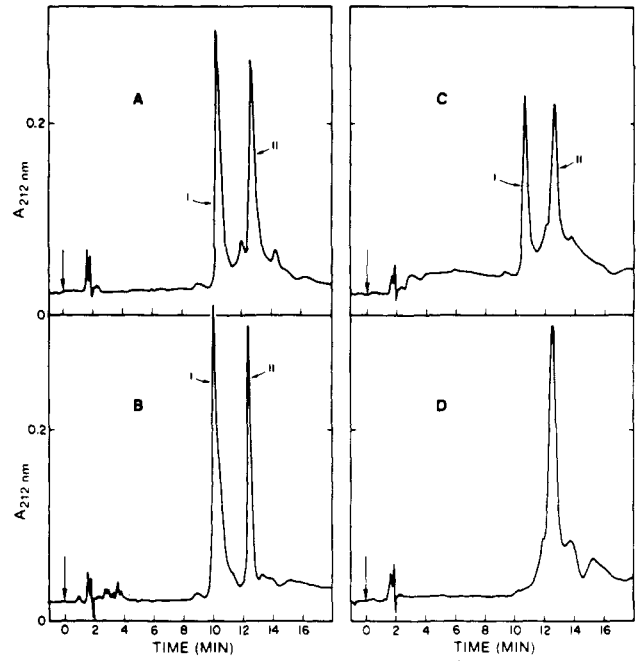


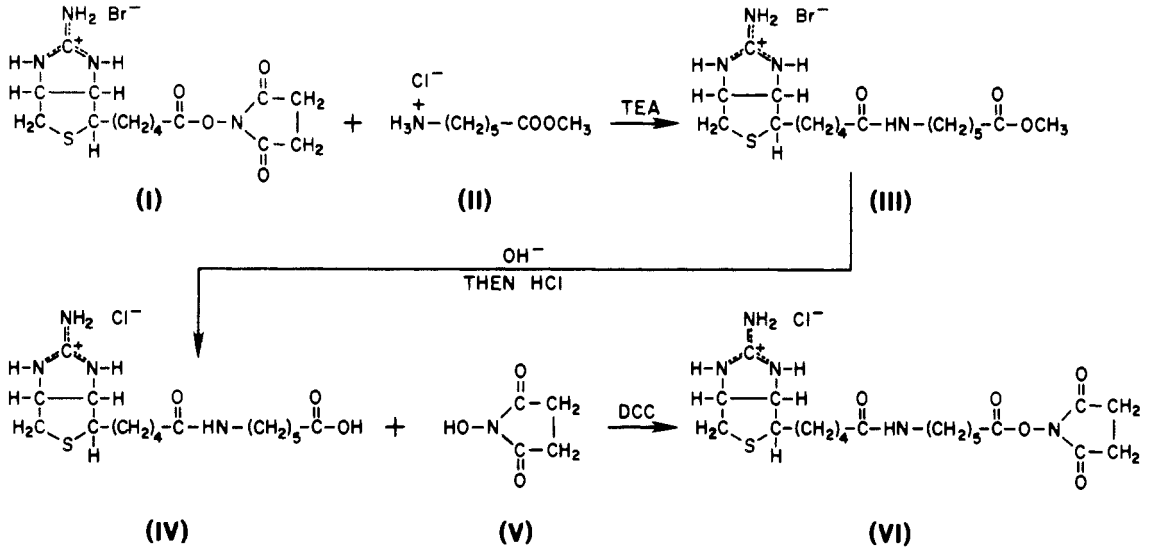
FIGURE 2: HPLC of porcine insulin and derivatives. (Panel A) Mixture of insulin (I) and N^{α,B^1}-[(dethiobiotinylamido)hexyl]insulin (II); (panel B) mixture of insulin (I) and N^{α,B^1}-biotinylinsulin (II); (panel C) mixture of insulin (I) and N^{α,B^1}-dethiobiotinylinsulin (II); (panel D) mixture of N^{α,B^1}-dethiobiotinylinsulin and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin. A Bondapak C_{18} column with solvent system; (pump A) 0.1% H_3PO_4; (pump B) 50% acetonitrile in 0.1% H_3PO_4 was employed. The gradient was from 50% to 80% pump B over 15 min with a pumping speed of 2 mL/min. Samples (20 μg) were applied.

Results

The original synthesis of iminobiotin (Hofmann & Axelrod, 1950) involved conversion of the sulfate salt of 5-(3,4-diaminothiophan-2-yl)pentanoic acid to the zwitterion with $Ba(OH)_2$ followed by reaction with cyanogen bromide. We have now developed a simpler procedure which circumvents the cumbersome $Ba(OH)_2$ step and affords the zwitterion of iminobiotin in one single operation with excellent yield. N^{α,B^1}-Iminobiotinylinsulin was prepared from $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin and the *N*-hydroxysuccinimido ester of iminobiotin hydrobromide as described for the synthesis of N^{α,B^1}-biotinylinsulin (Hofmann et al., 1977). As concerns the synthesis of the other insulin derivatives, they were prepared from the appropriate *N*-hydroxysuccinimido esters and $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin essentially according to the method of Krail et al. (1975) by using an 8-fold excess of acylating reagent. The final purification by chromatography on DEAE-cellulose, used in the purification of N^{α,B^1}-biotinyl- and N^{α,B^1}-iminobiotinylinsulins, was omitted since it appeared unlikely that small impurities present in the ion-exchanged product would interfere significantly with receptor binding.

The synthetic route to *N*-hydroxysuccinimido 6-(iminobiotinylamido)hexanoate is illustrated in Scheme I. Succinimido iminobiotinate hydrobromide (I) served to acylate methyl 6-aminohexanoate hydrochloride (II), and the ensuing methyl ester (III) was saponified to give the corresponding acid which was isolated in the form of its crystalline zwitterion.

Scheme I: Synthetic Route to *N*-Hydroxysuccinimido 6-(Iminobiotinylamido)hexanoate



This acid in the form of the hydrochloride (IV) was converted to the *N*-hydroxysuccinimido ester (VI) by the DCC procedure. The ester could not be obtained in crystalline form; thus the crude amorphous reaction product was employed for acylation of $N^{\alpha,A^1},N^{\epsilon,B^{29}}$-$Boc_2$-insulin. The same route served to synthesize N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin. Attempts to prepare the necessary *N*-hydroxysuccinimido 6-(dethiobiotinylamido)hexanoate by the DCC procedure were unsuccessful. However, the desired active ester was obtained, in crystalline form and good yield, by the use of the transesterification procedure of Sakakibara & Inukai (1965).

The structure assignments of the various insulin derivatives are based on the method of synthesis and the fact that they differed from insulin in their elution times on HPLC. Elution patterns of porcine insulin, N^{α,B^1}-iminobiotinylinsulin, and N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin have been published (Finn & Hofmann, 1981). A comparison of the elution patterns of N^{α,B^1}-dethiobiotinylinsulin and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin with insulin is illustrated in Figure 2. It is apparent that the elution times of the derivatized insulins differ markedly from that of porcine insulin and that N^{α,B^1}-dethiobiotinylinsulin and N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin are not separable under the experimental conditions employed. It was surprising to find that the six-carbon hydrophobic chain of the N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin exerted no influence on the retention time. The same was true of the behavior of N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin and N^{α,B^1}-iminobiotinylinsulin. Acid hydrolysates of the dansylated compounds did not contain Phe, and acid hydrolyses of the materials containing a spacer arm liberated the theoretically expected amount of 6-aminohexanoic acid.

A series of experiments was conducted comparing the dissociation of the complex succinoylavidin–N^{α,B^1}-biotinyl insulin to a number of succinoylavidin–N^{α,B^1}-biotinylinsulin analogue complexes in the presence of an excess of biotin (off-reaction).

The degree of dissociation immediately after mixing at pH 9.0 was 77% for iminobiotin and 83% for N^{α,B^1}-[6-(iminobiotinylamido)hexyl]insulin. N^{α,B^1}-Iminobiotinylinsulin failed to form a complex with succinoylavidin at pH 9.0. These derivatives were labeled with [^{14}C]formaldehyde (Rice & Means, 1971) because the ^{125}I-labeled samples proved to be unstable above pH 9.0. At pH 7.6 the N^{α,B^1}-dethiobiotinylinsulin complex was 88% dissociated after mixing, and the dethiobiotin complex which dissociates at a slower rate was

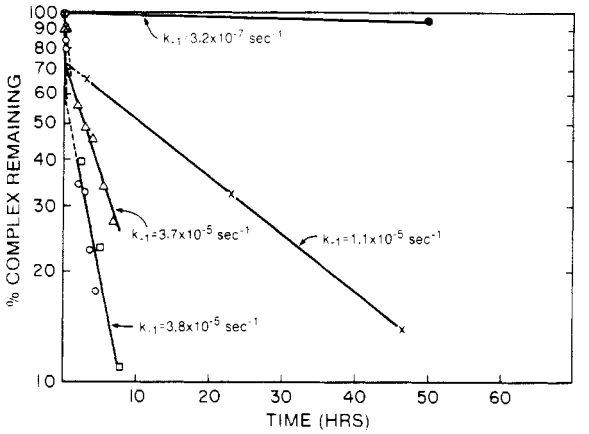


FIGURE 3: Rate of dissociation of A–B complexes. Succinoylavidin–biotin (●), succinoylavidin–dethiobiotin (×), succinoylavidin–N^{α,B^1}-biotinylinsulin (○), avidin–N^{α,B^1}-biotinylinsulin (□), succinoylavidin–N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin (△).

8.5% dissociated under the same conditions.

The rate of dissociation of a number of biotin analogue complexes is illustrated in Figure 3. The half-time for dissociation of biotin from avidin has been measured by Green (1975) as 200 days. We have determined the half-time dissociation of biotin from succinoylavidin as 127 days (Finn et al., 1980), indicating that extensive succinoylation of avidin does not alter significantly its biotin binding characteristics. Most surprising was the observation that the avidin and succinoylavidin complexes of N^{α,B^1}-biotinylinsulin dissociate rather readily. The N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin complex dissociates somewhat slower than the N^{α,B^1}-biotinylinsulin complex. The results of these dissociation studies in solution suggested that N^{α,B^1}-biotinylinsulin and some of its analogues would bind noncovalently to AH-Sepharose-immobilized succinoylavidin and could be retrieved biospecifically by exposure of the loaded resins to buffers containing biotin. Three compounds, i.e., N^{α,B^1}-biotinylinsulin, N^{α,B^1}-dethiobiotinylinsulin, and N^{α,B^1}-[6-(dethiobiotinylamido)-hexyl]insulin, were investigated. These materials were labeled with ^{125}I, and solutions of the radioactive compounds were rotated with AH-Sepharose-immobilized succinoylavidin. The resins thus loaded were subjected to exhaustive washing, and the amount of the attached ligand was determined. The washed resin was then rotated with buffer containing 20 mM biotin for ligand retrieval. The results are summarized in Table

Table I: Binding and Retrieval of Ligands from Affinity Column

ligand[a]	applied (pmol)	washings (pmol)	bound[c] (pmol)	biotin eluate[d] (pmol)
I	180	24	156 (87)	64 (41)
I	360	63	297 (83)	70 (24)
I	360[b]	57	303 (84)	57 (19)
I	720	118	602 (84)	160 (27)
II	180	104	76 (42)	42 (55)
II	360	198	162 (45)	76 (47)
III	180	28	152 (84)	56 (37)
III	360	80	280 (78)	98 (35)
III	360[b]	67	293 (81)	74 (25)

[a] (I) N^{α,B^1}-Biotinylinsulin; (II) N^{α,B^1}-dethiobiotinylinsulin; (III) N^{α,B^1}-[6-(dethiobiotinylamido)hexyl]insulin. Except where noted otherwise, the experiments were conducted at room temperature. [b] Experiment conducted at 4 °C. [c] Figures in parentheses are percent of added ligand retained. [d] Biotin eluate refers to picomoles recovered in the first 2 mL of eluate. Figures in parentheses represent percent of retained ligand recovered in first eluate. See materials and Methods for details.

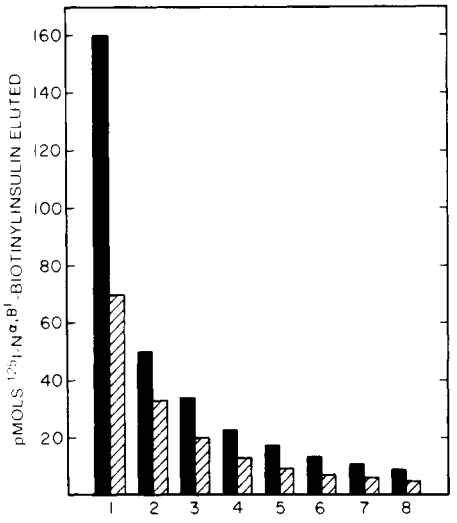


FIGURE 4: Elution of biotinylinsulin from affinity resin with 20 mM biotin. (Solid bars) Resin contained 720 pmol of biotinylinsulin. (Hatched bars) Resin contained 360 pmol of biotinylinsulin. (Abscissa) Successive 2-mL elutions of affinity resin.

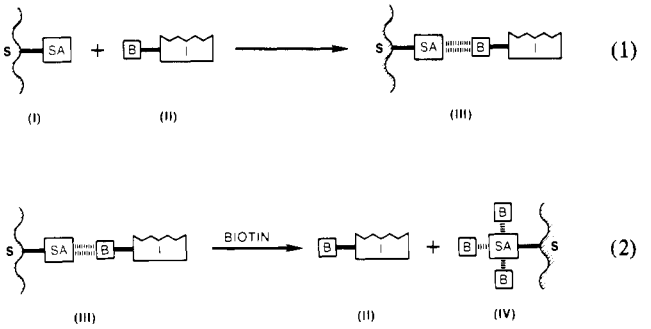
I. It is apparent that all three compounds bind to the resin, but N^{α,B^1}-dethiobiotinylinsulin is retained to a lesser degree than the other compounds. Elution with biotin-containing buffer specifically displaces a fraction of the bound ligand. From an inspection of Figure 4 it can be seen that the bulk of the ligand is displaced with the first 2 mL of biotin eluate. However, further elution brings off additional ligand. Successive elution of a resin which retained 602 pmol of N^{α,B^1}-biotinylinsulin afforded a 53% recovery of ligand. Similarly elution of a column that retained 297 pmol of the same ligand afforded a 55% recovery.

At pH 9.0 N^{α,B^1}-iminobiotinylinsulin was not retained by the succinoylavidin resin; N^{α,B^1}-[(iminobiotinylamido)hexyl]-insulin was retained, but most of it was removed on extensive washing with pH 9.0 application buffer (experiments not shown).

Discussion

The problem to be addressed in the present investigation concerns the development of affinity resins allowing the retrieval of insulin receptor under mild, biospecific conditions. Rather than attempt the direct removal of adsorbed receptor by cleavage of the insulin–receptor bond, it appeared advantageous to develop resins that are dissociable by biotin with liberation of complexes composed of insulin receptor attached to biotinylinsulin or analogues thereof. Scheme II illustrates our approach.

Scheme II: Formation and Dissociation of Biotinylinsulin Affinity Resin



First an affinity resin is prepared in which succinoylavidin is linked covalently to AH-Sepharose 4B (I). We employ succinoylavidin rather than avidin in order to minimize nonspecific interactions. We have shown that avidin binds avidly and nonsaturably to rat liver plasma membranes and that succinoylation of the protein drastically reduces these nonspecific interactions (Finn et al., 1980). The affinity resin (III) is obtained when N^{α,B^1}-biotinylinsulin or an analogue (II) is noncovalently attached to I. The assembly of the affinity resins (eq 1) presented no difficulties. Since we attach the biotin or biotin analogues to insulin in a targeted manner exclusively at the N terminus of the B chain, it was to be expected that the derivatized insulins would bind to the insulin receptors with essentially the same affinity ($K_D \sim 10^{-10}$ M) as native insulin, and indeed, we have observed that affinity resins of type III (B = biotin) bind solubilized insulin receptor from human placenta. The specificity of this interaction followed from the observation that control resins of type I failed to bind receptor (Finn & Hofmann, 1981). Desirable affinity resins (III) are those in which the SA–B bond is stable enough to withstand extensive washing but is dissociable by biotin with regeneration of a mixture of II plus IV (eq 2). In this step the available biotin binding sites on I become saturated with biotin.

Green (1966) investigated the thermodynamics of the binding of iminobiotin to avidin and found that the avidin–iminobiotin dissociation is pH dependent. The iminobiotin affinity for avidin approaches that of biotin at high pH values but is low at pH 4.0. We have exploited this property of iminobiotin for the isolation of streptavidin from the culture broth of *Streptomyces avidinii* (Hofmann et al., 1980). On the basis of our success with streptavidin isolation, we explored the potential of the iminobiotin–succinoylavidin system as a tool for insulin receptor isolation. At pH 9.0 the dissociation of the avidin–iminobiotin complex ($K_D \sim 10^{-8}$ M) is several orders of magnitude greater then that of the avidin–biotin complex ($K_D \sim 10^{-15}$ M). Thus the possibility had to be considered that modifications of the carboxyl group of the weaker binding iminobiotin would alter its affinity for avidin.

We have shown (Finn & Hofmann, 1981) that at pH 6.8 the dissociation constants of avidin complexes with iminobiotinylglycine, iminobiotinyl-L-phenylalanine, and 6-(iminobiotinylamido)hexanoic acid differed little from that of iminobiotin; i.e., modifications at the carboxyl group of iminobiotin had little influence on its binding to avidin. Even attachment of the bulky aromatic ring of phenylalanine did not disturb the interaction of iminobiotin with its avidin binding site. The avidin–N^{ϵ}-iminobiotinyllysine amide complex exhibited a significantly different dissociation behavior. Re-

pulsion of the positively charged α-amino group of lysine by the positively charged avidin may provide the explanation for this result. Syntheses of the compounds are described under Materials and Methods.

The observations that N^{α,B^1}-iminobiotinylinsulin fails to bind to succinoylavidin in solution or to immobilized succinoylavidin and the findings that N^{α,B^1}-[6-(iminobiotinylamido)hexyl]-insulin binds to AH-Sepharose 4B immobilized succinoylavidin but is eluted by extensive washing with a pH 9.0 buffer indicate that iminobiotin is not a desirable ligand for insulin receptor isolation. At first we were intrigued by the potential of the iminobiotin–succinoylavidin system as a tool for receptor retrieval; however, the weak affinity of iminobiotin for succinoylavidin, its sensitivity to steric effects (attachment of insulin), and the rather unphysiological hydrogen ion concentrations (pH 11.0) required for maximal binding argue against its use. In a recent paper, Orr (1981) proposed the use of this system as a tool for the retrieval of plasma membrane constituents.

A number of conclusions may be drawn from the results of measurements of the dissociation of biotin and dethiobiotin–succinoylavidin complexes in the presence of biotin (off-reaction). Of major significance was the observation that the attachment of insulin to biotin or dethiobiotin exerts a marked effect on the off-reaction rate (Figure 3). The half-time for dissociation of the biotin–succinoylavidin complex is 127 days. On the basis of the results shown in Figure 3, the half-time for dissociation of N^{α,B^1}-biotinylinsulin from avidin or succinoylavidin is approximately 1 h. This is due to the biphasic nature of the dissociation curve. This behavior, first reported by Green (1963), is not the result of succinoylation since it is also observed with avidin. Green reported that approximately 4% of the [^{14}C]biotin exchanged rapidly from avidin, and he attributed this behavior to partial denaturation of the avidin. It should be noted (Figure 3) that the rapid-exchange rate for N^{α,B^1}-biotinylinsulin which accounted for approximately 40% of the total exchange was the same regardless of whether avidin or succinoylavidin was employed. Immediately after being mixed, the dethiobiotin complex is 8.5% dissociated and is thus more stable than the N^{α,B^1}-dethiobiotinylinsulin complex which is 88% dissociated. The most obvious explanation for these findings is that attachment of insulin to biotin or biotin analogues imposes steric impediment to the interaction of the biotin residues with their avidin or succinoylavidin binding sites. Furthermore this explanation would predict that insertion of a spacer between the avidin binding site and the insulin molecule would increase the stability of the complexes. This effect was observed with both iminobiotin and dethiobiotin when the spacer 6-aminohexanoic acid was used.

Green et al. (1971) attempted to measure the depth of the biotin binding site on avidin by preparing a number of bifunctional reagents in which biotin molecules were attached via their carboxyl groups to both ends of spacer chains of varying lengths. From the influence of the length of the spacers on the ability of the reagents to form avidin polymers, he drew some conclusions about the size of the biotin binding cavity. When fewer than 12 bonds separated the carboxyl groups of the two biotins, no polymers were formed. Polymers were possible with 12, 13, or 14 bonds between, but these were relatively unstable. A chain containing 15 bonds was ideal. On the basis of these results, he concluded that the carboxyl group of the bound biotin must be buried about 9 Å below the surface of the avidin molecule. In our case this would mean that approximately 2–3 of the N-terminal amino acids of the B chain of biotinylinsulin would be inside the binding site. However, if Green's conclusions were totally correct, the binding of avidin to biotin in enzymes where biotin is attached to the ϵ-amino group of lysine would also not be favorable since the side chain of lysine has only six bonds separating it from the backbone of the peptide chain. This argument fails to consider the weakening effect repulsion arising from juxtaposing 2 highly positively charged avidin molecules must have on this system.

Clearly the insulin-derivatized biotins form much less stable complexes with avidin or succinoylavidin than do biotin or its analogues. The effect of attaching biotin to peptide hormones on the stability of avidin complexes will likely have to be determined on a case by case basis. The results of preliminary experiments with biotin derivatives of ACTH have indicated that it is not possible to generalize strictly from the insulin results.

The derivatives reported in this paper appear to fulfill all the criteria necessary for their application as ligands for biospecific affinity chromatography of insulin receptors: (a) The succinoylavidin–biotinylinsulin complexes interact well with the receptors (Finn et al., 1980), (b) biotinylinsulin and [6-(dethiobiotinylamido)hexyl]insulin form stable ligands with Sepharose-immobilized succinoylavidin, and (c) these ligands can be removed biospecifically with biotin.

References

Finn, F. M., & Hofmann, K. (1981) *Pept.: Synth., Struct. Funct., Proc. Am. Pept. Symp., 7th*, 1–18.

Finn, F. M., Kim, J. J., & Hofmann, K. (1979) *Biochem. Biophys. Res. Commun. 88*, 1153–1157.

Finn, F. M., Titus, G., Montibeller, J. A., & Hofmann, K. (1980) *J. Biol. Chem. 255*, 5742–5746.

Finn, F. M., Iwata, N., Titus, G., & Hofmann, K. (1981) *Z. Phys. Chem. (Leipzig) 362*, 679–684.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.

Green, N. M. (1963) *Biochem. J. 89*, 585–591.

Green, N. M. (1966) *Biochem. J. 101*, 774–779.

Green, N. M. (1975) *Adv. Protein Chem. 29*, 85–133.

Green, N. M., Konieczny, L., Toms, E. J., & Valentine, R. C. (1971) *Biochem. J. 125*, 781–791.

Hofmann, K., & Axelrod, A. E. (1950) *J. Biol. Chem. 187*, 29–33.

Hofmann, K., & Kiso, Y. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3516–3518.

Hofmann, K., Melville, D. B., & du Vigneaud, V. (1941) *J. Biol. Chem. 141*, 207–214.

Hofmann, K., Finn, F. M., Friesen, H.-J., Diaconescu, C., & Zahn, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2697–2700.

Hofmann, K., Finn, F. M., & Kiso, Y. (1978) *J. Am. Chem. Soc. 100*, 3585–3590.

Hofmann, K., Wood, S. W., Brinton, C. C., Montibeller, J. A., & Finn, F. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4666–4668.

Krail, G., Brandenburg, D., & Zahn, H. (1975) *Macromol. Chem. Suppl. 1*, 7–22.

Melville, D. B., Dittmer, K., Brown, G. B., & du Vigneaud, V. (1943) *Science (Washington, D.C.) 98*, 497–499.

Orr, G. A. (1981) *J. Biol. Chem. 256*, 761–766.

Rice, R. H., & Means, G. E. (1971) *J. Biol. Chem. 246*, 831–832.

Sakakibara, S., & Inukai, N. (1965) *Bull. Chem. Soc. Jpn. 38*, 1979–1984.