# Avidin Binding of Biotinylated Corticotropins†

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ABSTRACT: In order to assess the effect on avidin binding of attaching peptide hormones to biotin and biotin analogues, we have prepared [25-IBct]- and [25-DTBct]-$ACTH_{1-25}$ amides and have evaluated their steroidogenic activity and the rate of dissociation of their complexes with succinoylavidin. The analogues were assembled from available fragments of established structure by conventional procedures and were shown to be apparently homogeneous by high-pressure liquid chromatography, thin-layer chromatography, disc gel electrophoresis, and amino acid composition of acid hydrolysates. However, these approaches cannot provide information pertaining to the state of the Trp residue, which, due to the use of $F_3CCOOH$ for deprotection, may have become partially tert-butylated. The steroidogenic activity of the analogues, assessed with bovine adrenocortical cells, was within the limits of error of the assay identical with that of $ACTH_{1-24}$. Complexes with succinoylavidin were prepared from [25-Bct]- and [25-DTBct]-$ACTH_{1-25}$ amides and [25-DTBct]-$ACTH_{11-25}$ amide, and their dissociation rates were determined by the [$^{14}$C]biotin technique. At pH 9.0 [25-IBct]-$ACTH_{1-25}$ amide failed to form a complex with succinoylavidin. The rate of dissociation of the biocytin (Bct) analogue ($k_{-1} = 4 \times 10^{-7}$ $s^{-1}$) was found to be similar to that of the biotin–succinoylavidin complex ($k_{-1} = 6 \times 10^{-8}$ $s^{-1}$) [Hofmann, K., Titus, G., Montibeller, J. A., & Finn, F. M. (1982) *Biochemistry 21*, 978–984]. The rates of dissociation of the complexes of [25-DTBct]-$ACTH_{11-25}$ amide ($k_{-1} = 5.1 \times 10^{-5}$ $s^{-1}$) and [25-DTBct]-$ACTH_{1-25}$ amide ($k_{-1} = 9.1 \times 10^{-5}$ $s^{-1}$) are very similar to that of the dethiobiotin–succinoylavidin complex ($k_{-1} = 1.1 \times 10^{-5}$ $s^{-1}$) [Hofmann, K., Titus, G., Montibeller, J. A., & Finn, F. M. (1982) *Biochemistry 21*, 978–984]. It is concluded that the attachment of $ACTH_{1-24}$ to biocytin amide and its analogues exerts little influence on the affinity for avidin or succinoylavidin, a result that differs markedly from that obtained with biotinylinsulin–succinoylavidin complexes [Hofmann, K., Titus, G., Montibeller, J. A., & Finn, F. M. (1982) *Biochemistry 21*, 978–984]. The attachment of insulin to biotin weakens significantly the avidin–biotin interaction presumably because the insulin molecule with its stable conformation exerts a steric impediment. Such an effect is not observed with the smaller, flexible biotinylated $ACTH_{1-24}$ molecules.

Although many groups, including our own, have demonstrated specific binding of ACTH and ACTH fragments to adrenal cortical cells and membranes with concomitant activation of adenyl cyclase and protein kinases, nobody, to our knowledge, has succeeded in isolating homogeneous ACTH receptors. The high-affinity binding sites identified in adrenal cortical cells have as yet escaped detection in membranes, and the possibility cannot be overlooked that ACTH receptors undergo changes during membrane preparation. The search for high-affinity binding material in adrenal cortical membrane preparations has been uniformly unsuccessful. It would appear that less orthodox approaches have to be devised to identify these structures, and to this end, we are systematically investigating affinity columns in which biotinylated ACTH is attached noncovalently to immobilized avidin, succinoylavidin, or streptavidin as tools for the retrieval of ACTH receptors.

In a previous paper (Hofmann et al., 1982), we have described studies on the dissociation of succinoylavidin–biotinylinsulin complexes and observed that in contrast to succinoylavidin–biotin such complexes dissociate readily. It was concluded that the attachment of the insulin molecule to the carboxyl group of biotin exerts a steric impediment that weakens the succinoylavidin–biotin interaction. As a result, it became of considerable interest to investigate the dissociation behavior of other biotinylated peptide hormones such as ACTH. Previously (Hofmann & Kiso, 1976; Hofmann et al., 1978), we have attached biocytin amide (Bct-amide)[1] to the C terminus of $ACTH_{1-24}$ (Synacthen) and observed that this modification does not affect steroidogenic activity in isolated beef adrenal cortical cells. We have now prepared the amides of 25-iminobiocytin- (25-IBct-) and of 25-dethiobiocytin- (25-DTBct-) $ACTH_{1-25}$ and have determined their steroidogenic activity and the dissociation behavior of their complexes with succinoylavidin. Simplified structures of these compounds are illustrated in Figure 1.

## Experimental Procedures

*Materials and General Methods.* Biotin was obtained from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, NJ; [$^{14}$C]biotin, sp act. 48 mCi/mmol, was from Amersham. $N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-OH, H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu$^t$ and $ACTH_{1-24}$ (Synacthen) were gifts from Dr. W. Rittel, Ciba-Geigy Corp., Basel, Switzerland. CMC (carboxymethylcellulose) was from Bio-Rad Corp., Richmond, CA, Amberlite IRA-400 was from Mallinckrodt Chemical Works, St. Louis, MO, and di-*tert*-butyl dicarbonate was purchased from Fluka Corp., Buchs, Switzerland.

Thin-layer chromatography (TLC) was performed on glass plates coated with silica gel G (type 60) (E. Merck and Co., Darmstadt, German Federal Republic) in the following solvent systems: ($R_f^{I}$) 1-butanol–glacial acetic acid–water, 60:20:20; ($R_f^{II}$) chloroform–methanol–water, 8:3:1 (lower phase); ($R_f^{III}$) 1-butanol–pyridine–glacial acetic acid–water, 30:20:6:24. Compounds were visualized on thin-layer plates by fluorescamine (Udenfriend et al., 1972), the chlorine reagent (ex-

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[1] Abbreviations: Bct, biocytin; IBct, iminobiocytin; DTBct, dethiobiocytin; Synacthen, synthetic $ACTH_{1-24}$ (Ciba-Geigy); DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; DIPEA, *N*-ethyldiisopropylamine; TFA, trifluoroacetic acid or trifluoroacetyl; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; CCD, counter-current distribution; Tris, tris(hydroxymethyl)aminomethane; TEA, triethylamine.

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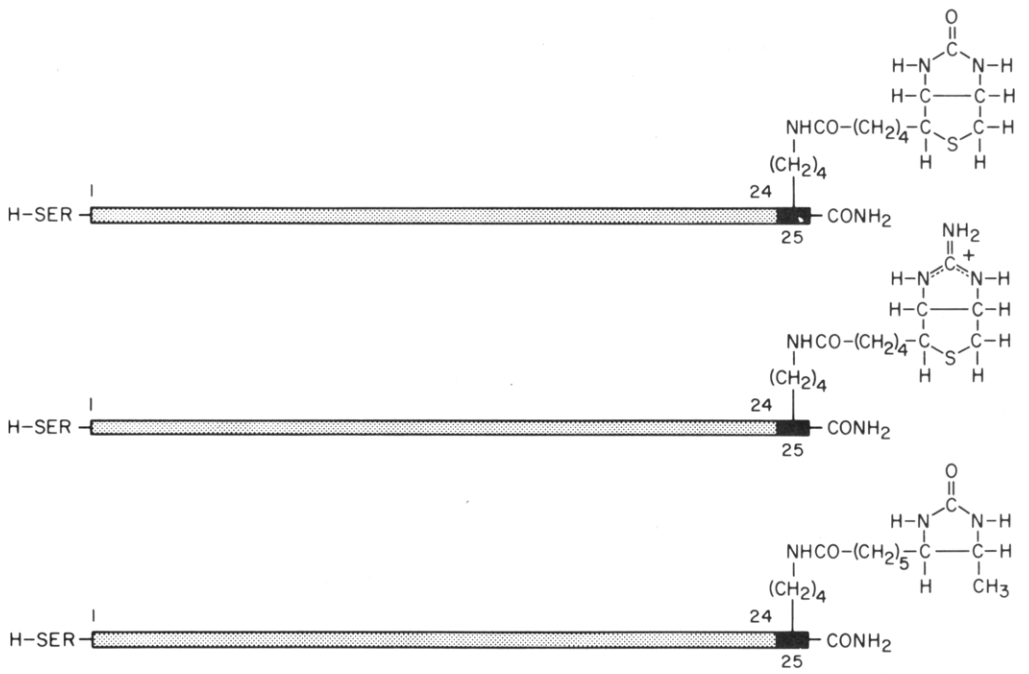


FIGURE 1: Simplified structures of [25-Bct]-ACTH$_{1-25}$ amide (top), [25-IBct]-ACTH$_{1-25}$ amide (middle), and [25-DTBct]-ACTH$_{1-25}$ amide (bottom).

posure to hypochlorite followed by spraying of the dried plates with a 1:1 mixture of 0.4% KI and 1% starch solution in water), and *p*-(dimethylamino)cinnamaldehyde (McCormick & Roth., 1970). Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, NJ. High-pressure liquid chromatography (HPLC) was performed with a Waters system equipped with a Model 660 solvent programmer. For amino acid analyses, samples were hydrolyzed with constant boiling HCl containing 0.1% phenol at 110 °C for 24 h. Trifluoroacetates and tosylates were converted to the corresponding acetates on acetate cycle Amberlite IRA-400 with dilute acetic acid as the solvent. Adrenal cell assays were performed essentially as described (Finn et al., 1976).

*Ligand Binding and Displacement Studies.* Complexes were prepared by mixing succinoylavidin (Finn et al., 1980) (1 equiv) in 50 mM Tris-HCl, pH 7.6, with the desired ligand (8 equiv; 2-fold excess on a molar basis) in 0.01 N HCl (4 mg/mL), and excess ligand was removed by gel filtration on Sephadex G-50 with 50 mM Tris-HCl, pH 7.6, as the solvent. The concentration of the complex in the high molecular weight eluates was determined spectrophotometrically, and a 100-fold excess (based on succinoylavidin) 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 high molecular weight eluates provided a measure of the rate of dissociation.

*Synthetic Aspects.* (*A*) *Iminobiocytin* (*IBct*) *Amide Ditosylate.* TEA (0.138 mL, 1 mmol) was added to a solution of $N^{\alpha}$-Boc-L-lysine amide (prepared from 379 mg, 1 mmol, of $N^{\alpha}$-Boc-$N^{\epsilon}$-Z-lysine amide) (Hofmann et al., 1978) and *N*-hydroxysuccinimido iminobiotinate (Orr, 1981; Hofmann et al., 1982) (463 mg, 1.1 mmol) in DMF (6 mL), and the solution was stirred at room temperature for 24 h. The solvent was evaporated, and the residue was triturated with ethyl acetate, collected, washed with ethyl acetate, and dried; yield 688 mg.

This product was deprotected with 90% TFA (10 mL), and TFA ions were exchanged for acetate ions to give 509 mg of crude product. This material was dissolved in water (100 mL), and the solution (50 mL) was applied to a CMC column (2.2 × 14 cm) (acetate cycle) that was eluted first with water (350 mL) and then in stepwise manner with 0.015, 0.03, and 0.06% acetic acid. Fractions (20 mL each) were collected and monitored by the chlorine reaction. Fractions containing the desired material (0.03% acetic acid eluates) were pooled, concentrated to a small volume in vacuo, and lyophilized: yield (two batches) 392 mg (80%); mp 106–108 °C; $[\alpha]^{27}_{D}$ +45.0° (*c* 0.56, 5% acetic acid); $R_f^{I}$ 0.1, $R_f^{III}$ 0.4. A sample for analysis was dried in vacuo at 80 °C. Anal. Calcd for $C_{16}H_{30}N_6O_2S{\cdot}2CH_3COOH$: C, 49.0; H, 7.8; N, 17.1. Found: C, 48.6; H, 7.7; N, 17.2. The acetate salt was converted to the ditosylate salt (Brundish & Wade, 1973).

(*B*) *Dethiobiocytin* (*DTBct*) *Amide Tosylate.* DIPEA (0.34 mL, 2 mmol) was added to a solution of $N^{\alpha}$-Boc-L-lysine amide (prepared from 760 mg, 2 mmol, of $N^{\alpha}$-Boc-$N^{\epsilon}$-Z-L-lysine amide) (Hofmann et al., 1978) and *N*-hydroxysuccinimido dethiobiotinate (Hofmann et al., 1982) (716 mg, 2.3 mmol) in DMF (10 mL), and the solution was stirred at room temperature for 20 h. The solvent was evaporated, and the residue was precipitated with ethyl acetate, washed with ethyl acetate, and dried; yield 610 mg. For purification, the compound was precipitated from methanol with ethyl acetate–ether (1:1) and dried; yield 554 mg (62%); mp 93–94 °C; $[\alpha]^{25}_{D}$ +7.2° (*c* 1.203, methanol); $R_f^{I}$ 0.5, $R_f^{II}$ 0.4, $R_f^{III}$ 0.7. A sample for analysis was dried in vacuo at 100 °C (melt). Anal. Calcd for $C_{21}H_{39}N_5O_5$: C, 57.1; H, 8.9; N, 15.9. Found: C, 56.9; H, 9.0; N, 15.7. The above product (200 mg) was deprotected with 90% $F_3CCOOH$ (3 mL), and $F_3CCOOH$ ions were exchanged for acetate ions; yield 174 mg (96%). The acetate salt was converted to the tosylate salt (Brundish & Wade, 1973); yield 260 mg.

(*C*) *H-Lys*(*Boc*)*-Pro-Val-Gly-Lys*(*Boc*)*-Lys*(*Boc*)*-Arg-Arg-Pro-Val-Lys*(*Boc*)*-Val-Tyr-Pro-IBct Amide Tetratosylate.* $N^{\alpha}$-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH ditosylate (Hofmann et al., 1978) (463 mg, 0.186 mmol), IBct amide ditosylate (265 mg, 0.362 mmol), and 1-hydroxybenzotriazole hydrate (164 mg, 1.074 mmol) were dissolved in DMF–pyridine (2:1) (12 mL), and DCC (222 mg, 1.074 mmol) in DMF (3 mL) was

added in three equal portions over a span of 2 h with stirring. The mixture was stirred for 40 h at room temperature, the bulk of the solvents was evaporated, and the residue was precipitated with ethyl acetate, washed with ethyl acetate, and dried. The material was dissolved in 50% acetic acid (20 mL), and tosylate ions were exchanged for acetate ions; yield 583 mg. A solution of this material in 50% methanol was then chromatographed on a CMC column (2.8 × 9.5 cm) in the manner described previously (Hofmann et al., 1978). The desired material was eluted with the 0.03% acetic acid: yield 309 mg (65%); $[\alpha]^{27}_D$ −54.5° (*c* 0.568, methanol); $R_f^I$ 0.2, $R_f^{III}$ 0.6. This product (304 mg) was deprotected with piperidine in the manner described previously (Hofmann et al., 1978): yield 292 mg; $[\alpha]^{27}_D$ −21.6° (*c* 1.1, DMF); $R_f^I$ 0.2, $R_f^{II}$ 0.6. The compound was converted to the tetratosylate (Brundish & Wade, 1973).

(*D*) *H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-IBct Amide Heptaacetate.* The above compound (15 mg) was deprotected with 90% TFA in the usual manner, and TFA ions were exchanged for acetate ions. Amino acid ratios in the acid hydrolysate were as follows: $Lys_{4.5}Arg_{2.2}Pro_{3.4}Gly_{1.2}Val_{2.8}Tyr_{0.9}$.

(*E*) *H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct Amide Tritosylate.* $N^{\alpha}$-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH ditosylate (Hofmann et al., 1978) (464 mg, 0.19 mmol), DTBct amide tosylate (260 mg, 0.50 mmol), and 1-hydroxybenzotriazole hydrate (170 mg, 1.11 mmol) were dissolved in DMF–pyridine (2:1) (9 mL), and DCC (229 mg, 1.11 mmol) dissolved in DMF (3 mL) was added in three equal portions over a span of 2 h with stirring. The mixture was stirred for 90 h at room temperature, the reaction mixture was cooled at 0 °C, and DCU was removed by filtration. The filtrate was evaporated, and the product was precipitated with ethyl acetate, washed with ethyl acetate, and dried; yield 665 mg. The product was dissolved in 50% acetic acid, and tosylate ions were exchanged for acetate ions; yield 590 mg. The acetate (295 mg) was dissolved in 50% aqueous methanol (150 mL), and the pH of the solution was adjusted to approximately 7.5 with TEA. The solution was applied to a CMC column (1.8 × 12 cm) that was eluted with 50% methanol (50 mL) followed by methanol–0.015% acetic acid (1:1) (200 mL) and methanol–0.03% acetic acid (1:1) (300 mL). Fractions (10 mL each) were collected and monitored by absorbance at 280 nm and TLC. Fractions 96–130 (0.03% acetic acid eluates) containing single-spot material ($R_f^I$ 0.4) were pooled, evaporated to a small volume, and lyophilized: yield 146 mg; $[\alpha]^{28}_D$ −63.6° (*c* 0.7, methanol); $R_f^I$ 0.4; amino acid ratios in acid hydrolysate, $Lys_{4.5}Arg_{2.1}Pro_{2.9}Gly_{1.2}Val_{3.2}Tyr_{1.2}$. This product (270 mg) was deprotected with piperidine in the manner described previously (Hofmann et al., 1978) (yield 254 mg) and converted to the tritosylate (Brundish & Wade, 1973).

(*F*) *$N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-IBct Amide Triacetate.* $N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-OH tosylate (182 mg, 0.112 mmol), H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-IBct amide tetratosylate (310 mg, 0.1 mmol), and 1-hydroxybenzotriazole monohydrate (61 mg, 0.4 mmol) were dissolved in DMF–pyridine (2:1) (12 mL), and DCC (124 mg, 0.6 mmol) in DMF (1.5 mL) was added in three equal portions over a span of 2 h with stirring. The mixture was stirred for 24 h at room temperature, and the reaction products were precipitated with ethyl acetate, washed with ethyl acetate, and dried; yield 508 mg. The material was dissolved in 50% acetic acid, and tosylate ions were exchanged for acetate ions; yield 444 mg. The acetate (408 mg) was dissolved in the lower phase of the solvent system methanol–ammonium acetate, pH 4.5–chloroform–carbon tetrachloride (8:4:5:2) (40 mL) (Schwyzer & Kappeler, 1963) and was subjected to 250 transfers in a Craig-Post counter-current machine. The contents of tubes 170–250 containing the desired material were pooled, concentrated to a small volume, and lyophilized. Ammonium acetate was sublimed off in vacuo at 40 °C, and the residue was lyophilized from dilute acetic acid: yield 290 mg (70%); $R_f^I$ 0.3, $R_f^{III}$ 0.7; amino acid ratios in acid hydrolysate, $Lys_{4.6}Arg_{3.0}His_{1.1}Ser_{2.0}Glu_{1.1}Pro_{3.3}Gly_{1.9}Val_{3.0}Met_{1.1}Tyr_{1.6}Phe_{1.2}$.

(*G*) *[25-IBct]-ACTH$_{1-25}$ Amide Octaacetate.* The above protected peptide amide (120 mg) was deprotected with 90% TFA containing 2% 1,2-ethanediol (5 mL), the solvent was evaporated, and the product was precipitated with ether, washed with ether, and dried. TFA ions were exchanged for acetate ions to give 116 mg of the crude acetate. This material in batches of 53 mg each was dissolved in 0.05 M phosphate buffer, pH 7.0 (3 mL), and the solution was applied to a column of sodium cycle CMC (0.9 × 12 cm) previously equilibrated with the same buffer. The column was eluted with application buffer (30 mL); then, a linear gradient (140 mL, 0–0.55 M) of sodium chloride was applied (Allen et al., 1979). Fractions (3 mL each) were collected and monitored for absorbance at 280 nm. Fractions containing the desired product were pooled, evaporated, and lyophilized. The resulting material was desalted on Sephadex G-25 with 10% acetic acid as the solvent. The salt-free material was reduced with thioglycolic acid (Hofmann et al., 1966). Five batches, processed similarly, were combined to give 186 mg (66%) of the final product. Amino acid ratios in the acid hydrolysate were as follows: $Lys_{4.8}His_{1.0}Arg_{3.1}Ser_{2.0}Glu_{1.2}Pro_{2.9}Gly_{2.1}Val_{2.8}Met_{1.2}Tyr_{1.9}Phe_{1.1}$.

(*H*) *$N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct Amide Tritosylate.* $N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly tosylate (163 mg, 0.1 mmol), H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct amide (257 mg, 0.09 mmol), and 1-hydroxybenzotriazole monohydrate (55 mg, 0.36 mmol) were dissolved in a mixture of DMF–pyridine (2:1) (12 mL), and DCC (111 mg, 0.54 mmol) in 1.5 mL of DMF was added in three equal portions over a span of 2 h with stirring. The mixture was stirred at room temperature for 22 h, and the reaction products were precipitated with ethyl acetate, washed with ethyl acetate, and dried; yield 416 mg. The desired product was isolated from the crude material by CCD as described for the iminobiotinyl derivative: yield 273 mg (70%); $R_f^I$ 0.4, $R_f^{III}$ 0.7; amino acid ratios in acid hydrolysate, $Lys_{4.7}His_{0.9}Arg_{2.8}Ser_{2.3}Glu_{1.2}Pro_{3.5}Gly_{2.0}Val_{2.6}Met_{1.1}Tyr_{1.9}Phe_{1.1}$.

(*I*) *[25-DTBct]-ACTH$_{1-25}$ Amide Heptaacetate.* The protected material (273 mg, 0.062 mmol) was deprotected with 90% TFA and purified in batches of 55 mg each as described for the preparation of the corresponding iminobiotin derivative: total yield 146 mg (44%); $R_f^{III}$ 0.6; amino acid ratios in acid hydrolysate, $Lys_{5.1}His_{1.1}Arg_{3.4}Ser_{1.9}Glu_{1.1}Pro_{2.8}Gly_{2.1}Val_{2.9}Met_{0.9}Tyr_{1.8}Phe_{1.0}$.

## Results

The synthetic route to [25-IBct]-ACTH$_{1-25}$ amide and [25-DTBct]-ACTH$_{1-25}$ amide is patterned along the lines of our previous synthesis of [25-Bct]-ACTH$_{1-25}$ amide (Hofmann

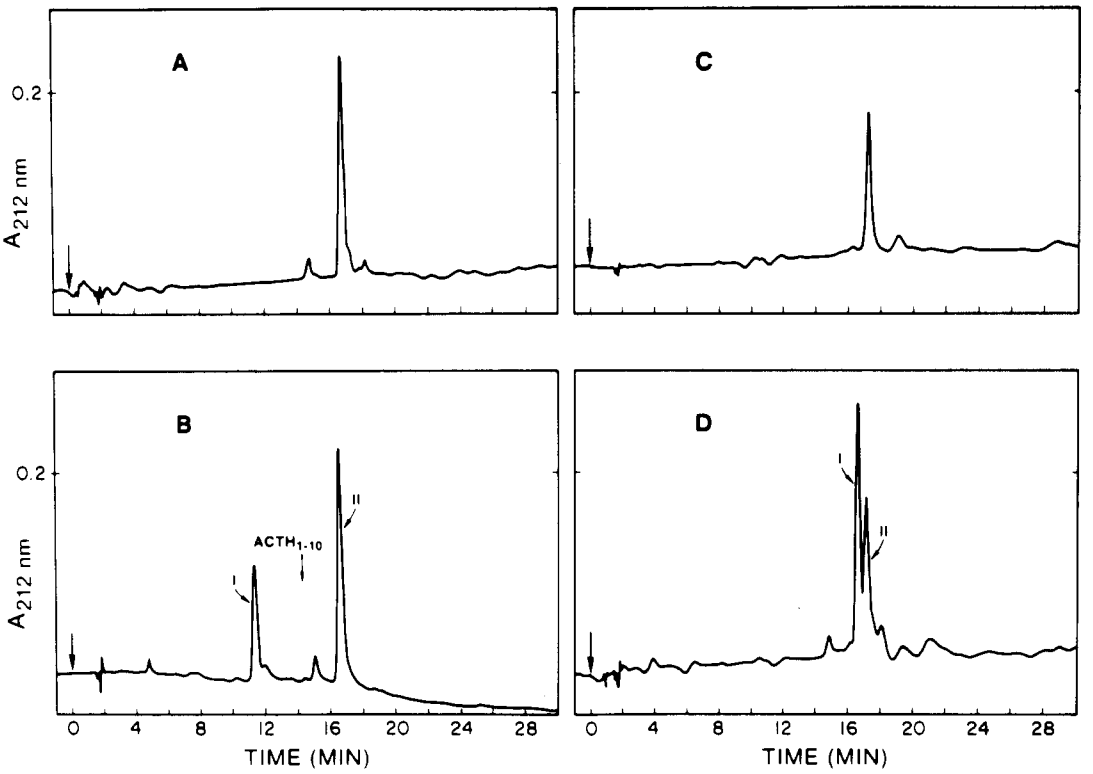


FIGURE 2: HPLC of $ACTH_{1-24}$ and derivatives: (panel A) $ACTH_{1-24}$ (Synacthen); (panel B) mixture of [25-DTBct]-$ACTH_{11-25}$ amide (I) and $ACTH_{1-24}$ (II); (panel C) [25-IBct]-$ACTH_{1-25}$ amide; (panel D) mixture of $ACTH_{1-24}$ (I) and [25-IBct]-$ACTH_{1-25}$ amide (II). A Bondapak $C_{18}$ column with the following solvent system was employed: (pump A) 0.05% TFA–(pump B) 50% acetonitrile in 0.05% TFA. The linear gradient was 20–80% pump B over 30 min with a pumping speed of 2 mL/min. Samples of approximately 10 $\mu$g were applied.

& Kiso 1976; Hofmann et al., 1978). Briefly, it involves N-trifluoroacetylation of H-Lys(Boc)-Pro-Val-Gly-Lys-(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu$^t$ followed by removal of the Boc groups and the OBu$^t$ ester with TFA. Tert-butylation of the deprotected material with di-*tert*-butyl dicarbonate resulted in substitution of the lysine $\epsilon$-amino groups, leaving the C-terminal carboxyl group unprotected. The resulting material, in the form of its tosylate, was then coupled to the tosylates of either IBct amide or DTBct amide by the DCC–HOBT procedure (König & Geiger, 1970). Following ion exchange and purification, the TFA protecting group was removed with piperidine (Ontjes & Anfinsen, 1969), and the ensuing products were acylated with Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-OH again by the DCC–HOBT method. The crude products were purified by CCD, deprotected with TFA containing ethane-1,2-dithiol, and, following ion exchange, chromatographed on CMC (Allen et al., 1979). The final products were incubated with thioglycolic acid to convert trace contamination by methionine sulfoxide to methionine (Hofmann et al., 1966). The homogeneity of the final materials and certain intermediates was evaluated by TLC, HPLC, disc gel electrophoresis, and amino acid analyses of acid hydrolysates. An inspection of the HPLC's of the biotinylated ACTH derivatives (Figures 2 and 3) demonstrates that [25-Bct]-, [25-IBct]-, and [25-DTBct]-$ACTH_{1-25}$ amides elute as single peaks that are retarded with respect to $ACTH_{1-24}$. Mixtures of $ACTH_{1-24}$ and the biotinylated species are readily separable. The starting materials used for the synthesis of the biotinylated compounds elute from the chromatograms well in advance of the desired products (Figure 2B) and are not seen on the chromatograms of the final products. These results suggest that the synthetic ACTH derivatives are homogeneous.

The amino acid composition of acid hydrolysates of the synthetic materials are in satisfactory agreement with theoretically expected values expect that the lysine recoveries are somewhat low. This may be the result of incomplete hydrolysis of the amide bond that links the biotin derivatives to the $\epsilon$-amino group of lysine. Disc gel electropherograms of the biotinylated ACTH derivatives revealed the presence of single diffuse bands (results not shown).

[25-IBct]- and [25-DTBct]-$ACTH_{1-25}$ amides are as effective as $ACTH_{1-24}$ in stimulating steroid production in isolated bovine adrenal cortical cells (Figure 4). These results confirm our previous finding that the addition of Bct-amide to the C terminus of $ACTH_{1-24}$ does not lower steroidogenic activity (Hofmann & Kiso, 1976).

The dissociation rate of the complexes of [25-Bct]- and [25-DTBct]-$ACTH_{1-25}$ amides as well as [25-DTBct]-$ACTH_{11-25}$ amide with succinoylavidin was determined with the results shown on Figure 5. The Bct analogue ($k_{-1} = 4 \times 10^{-7}\ s^{-1}$) has a dissociation rate similar to that of biotin itself ($6 \times 10^{-8}\ s^{-1}$). The rates of dissociation of the complexes of [25-DTBct]-$ACTH_{11-25}$ amide and [25-DTBct-$ACTH_{1-25}$ amide with succinoylavidin are similar to that of the dethiobiotin–succinoylavidin complex ($5.1 \times 10^{-5}\ s^{-1}$, $9.1 \times 10^{-5}\ s^{-1}$, and $1.1 \times 10^{-5}\ s^{-1}$, respectively).

## Discussion

Although the analytical results do not reveal the presence of contaminants in the new ACTH analogues, they fail to provide information regarding the state of the Trp residue that is destroyed during acid hydrolysis. It is by now well established that exposure of Trp-containing peptides to TFA brings about partial tert-butylation of the indole portion of this amino acid (Jaeger et al., 1978). Addition of ethane-1,2-dithiol to the TFA appears to decrease, but does not eliminate, this side reaction. The Trp residue in ACTH is important for steroidogenesis as its replacement by Phe or Ala (Hofmann et al., 1974; Finn et al., 1976) drastically lowers steroidogenic ac-

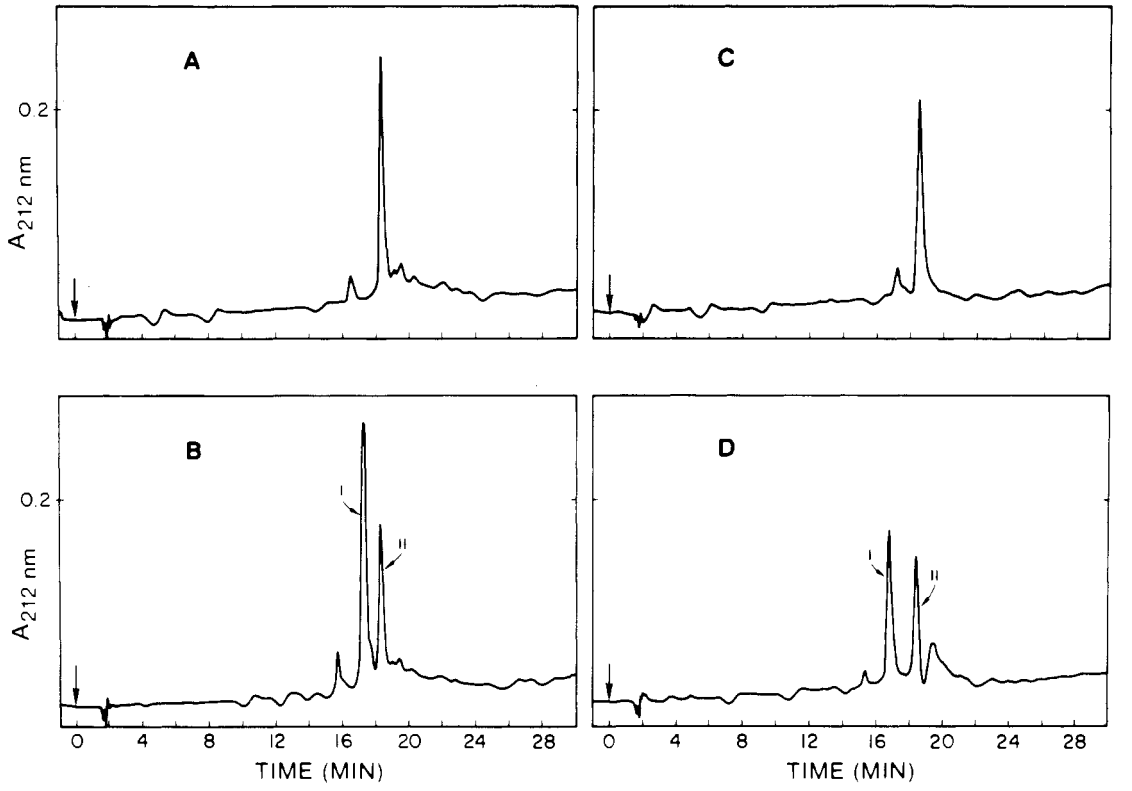


FIGURE 3: HPLC of $ACTH_{1-24}$ and derivatives: (panel A) [25-Bct]-$ACTH_{1-25}$ amide; (panel B) mixture of $ACTH_{1-24}$ (I) and [25-BCt]-$ACTH_{1-25}$ amide (II); (panel C) [25-DTBct]-$ACTH_{1-25}$ amide; (panel D) mixture of $ACTH_{1-24}$ (I) and [25-DTBct]-$ACTH_{1-25}$ amide (II). Conditions were those described for Figure 2.

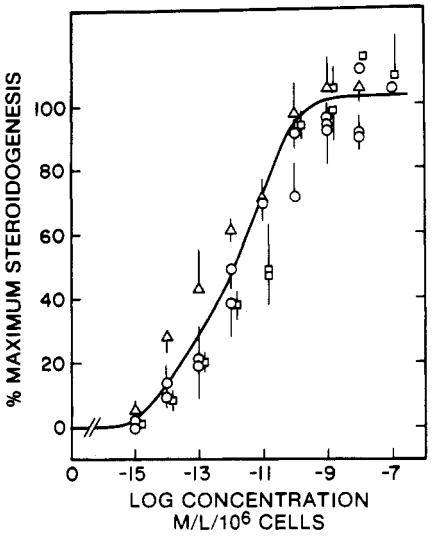


FIGURE 4: Stimulation of steroidogenesis in bovine adrenocortical cells by $ACTH_{1-24}$ (○), [25-IBct]-$ACTH_{1-25}$ amide (□), and [25-DTBct]-$ACTH_{1-25}$ amide (△). Stimulation was 5–10-fold above base line. Vertical bars indicate standard deviation.

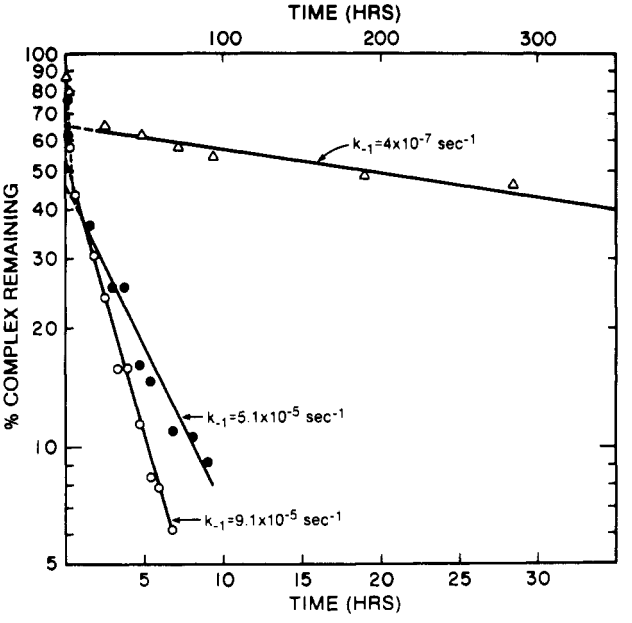


FIGURE 5: Rate of dissociation of A–B complexes: succinoylavidin–[25-Bct]-$ACTH_{1-25}$ amide (△) (time scale on top); succinoylavidin–[25-DTBct]-$ACTH_{11-25}$ amide (●); succinoylavidin–[25-DTBct]-$ACTH_{1-25}$ amide (○).

tivity. However, it appears that tert-butylation of Trp does not affect biological activity. The ACTH analogues [9-Trp-(1-Bu$^t$)]-$ACTH_{1-19}$ and [9-Trp(2,5,7-Bu$^t_3$)]-$ACTH_{1-19}$ are potent in the Sayers test for steroidogenicity (Löw et al., 1979). On the basis of these results, one may conclude that the steroidogenic activity of the synthetic compounds described would not be significantly affected by contamination with Trp tert-butylated materials. This conclusion is borne out by the results of biological testing.

In his extensive studies on the stability of avidin–biotin complexes, Green (1966) investigated avidin–iminobiotin and avidin–dethiobiotin complexes. Both complexes were found to dissociate more readily than the avidin–biotin complex, and the interaction of iminobiotin with avidin exhibited marked pH dependence. At pH 11.0, this compound bound firmly to avidin, but at pH 4.0, the complex was readily dissociated. This property of iminobiotin provided the key to the isolation of streptavidin from the culture fluid of *Streptomyces avidinii* (Hofmann et al., 1980). In this same study, we demonstrated that cycling between extremes of pH was not essential for function of iminobiotin affinity resins. Immobilized imino-

biotin retained $^{125}$I-labeled succinoylavidin derivatives applied at pH values as low as 7.5. These results seemed to indicate that iminobiotinylated hormones might be useful for reversible affinity chromatography of their receptors. Unfortunately [25-IBct]-ACTH$_{1-25}$ amide fails to form a complex with succinoylavidin even at pH 9.0.

Although we had previously shown (Hofmann et al., 1982) that $N^{\alpha,B1}$-(iminobiotinyl)insulin did not complex with succinoylavidin, the behavior of the corresponding ACTH derivative could not be predicted from these results since attachment of biotin or a biotin analogue to the N terminus of the B chain of insulin apparently severely interferes with the ability of biotin to interact with avidin. For example, $N^{\alpha,B1}$-biotinylinsulin–succinoylavidin complexes dissociate at a rate approximately 1000 times faster than their biotin–succinoylavidin counterparts. Only when an alkyl spacer arm is interposed between a biotin analogue and the insulin molecule does the dissociation behavior correspond to that of the unattached biotin congener itself as was shown for the dissociation rates of dethiobiotin vs. that of $N^{\alpha,B1}$-[6-[(dethiobiotinyl)amido]-hexyl]insulin.

In the case of ACTH, interference by the hormone with the binding of biotin or biotin analogues is less severe as evidenced by the fact that dissociation rates for succinoylavidin complexes of [25-Bct]-ACTH$_{1-25}$ and [25-DTBct]-ACTH$_{1-25}$ are of the same order of magnitude as for the corresponding underivatized biotins. Unlike insulin, where a well-defined three-dimensional conformation has been established, ACTH, which seems to possess little detectable conformation, is probably a more flexible molecule, and this may account for accessibility of biotin to its binding site.

The results of many studies [for a review, see Hofmann 1974)] have clearly established that the C-terminal portion of the ACTH molecule (from residue 21 to 39) is involved neither in binding to the receptor nor in the biological activity of the hormone–receptor complex. A molecule as small as ACTH$_{1-20}$ amide is essentially a full agonist whose biological activity differs little from that of ACTH$_{1-24}$ (also a full agonist) in all systems examined. Therefore, it was not surprising to find that the biocytin analogues of ACTH$_{1-24}$ described in this and a previous paper (Hofmann & Kiso, 1976) displayed a biological potency equal to that of ACTH$_{1-24}$. In a previous study, using rat adrenal cells (Finn et al., 1979), we have compared the steroidogenic activity of [25-Bct]-ACTH$_{1-25}$ amide (standard) to that of hormone–avidin complexes in which the ratio of hormone to avidin was 4:1, 1:1, and 0.1:1, respectively. Maximum steroidogenesis was elicited in every instance, and the log dose–response curves were parallel. The ED$_{50}$ value of the standard was 5.6 nM; those of the 4:1, 1:1, and 0.1:1 complexes were 9.0, 22.5, and 18 nM, respectively. The presence of an excess of avidin (0.1:1 ratio) did not interfere with the ability of the peptide to stimulate steroid production. The observation that the rate of dissociation of the [25-Bct]-ACTH$_{1-25}$ amide–succinoylavidin complex is as slow as that of biotin–succinoylavidin suggests that steroidogenic activity of the complex cannot be ascribed to the presence of dissociated Bct-ACTH molecules. Rather this information, coupled with our earlier finding that the complex is approximately as active as the uncomplexed hormone, strongly indicates that avidin does not significantly interfere with ACTH–receptor action.

**Registry No.** [25-IBct]-ACTH$_{1-25}$ amide octaacetate, 84118-43-4; [25-DTBct]-ACTH$_{1-25}$ amide heptaacetate, 84171-50-6; *N*-hydroxysuccinimido iminobiotinate, 84171-51-7; $N^{\alpha}$-Boc-L-lysine amide biotinate, 84118-44-5; IBct amide diacetate, 80750-28-3; IBct amide ditosylate, 84171-52-8; *N*-hydroxysuccinimidodethiobiotinate, 80750-24-9; $N^{\alpha}$-Boc-L-lysine amide dethiobiotinate, 84118-45-6; DTBct amide tosylate, 84118-47-8; H-Lys(Boc)-Pro-Val-Gly-Lys-(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-IBct amide tetratosylate, 84118-50-3; $N^{\alpha}$-TFA-Lys(Boc)-Pro-Val-Gly-Lys-(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-IBct amide, 84118-48-9; H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-IBct amide heptaacetate, 84118-52-5; H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct amide tritosylate, 84118-54-7; $N^{\alpha}$-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH ditosylate, 67917-61-7; $N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu-(OBu$^t$)-His-Phe-Arg-Trp-Gly-OH tosylate, 67917-67-3; $N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-IBct amide triacetate, 84281-70-9; $N^{\alpha}$-Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys-(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct amide tritosylate, 84281-71-0; $N^{\alpha}$-Boc-L-lysine amide, 58725-24-9.

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