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Radioactive Probes for Adrenocorticotropic Hormone Receptors†

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ABSTRACT: Our attempts to develop adrenocorticotropic hormone (ACTH) analogues that can be employed for ACTH receptor identification and isolation began with the synthesis of ACTH fragments containing N^{ϵ}-(dethiobiotinyl)lysine (dethiobiocytin) amide in position 25 to be used for affinity chromatographic purification of hormone–receptor complexes on Sepharose-immobilized avidin resins. Because labeling ACTH or ACTH fragments by conventional iodination techniques destroys biological activity due to oxidation of Met[4] and incorporation of iodine into Tyr[2], we have prepared [Phe[2],Nle[4]]$ACTH_{1-24}$, [Phe[2],Nle[4],biocytin[25]]$ACTH_{1-25}$ amide, and [Phe[2],Nle[4],dethiobiocytin[25]]$ACTH_{1-25}$ amide by conventional synthetic techniques. The HPLC profiles and amino acid analyses of the final products indicate that the materials are of a high degree of purity. The amount of tertiary butylation of the Trp residue in the peptides was assessed by NMR and was found to be less than 0.5%. All three peptides are equipotent with the standard $ACTH_{1-24}$ as concerns their ability to stimulate steroidogenesis and cAMP formation in bovine adrenal cortical cells. Iodination of [Phe[2],Nle[4]]$ACTH_{1-24}$, with iodogen as the oxidizing agent, has been accomplished without any detectable loss of biological activity. The mono- and diiodo derivatives of [Phe[2],Nle[4]]$ACTH_{1-24}$ have been prepared, separated by HPLC, and assayed for biological activity. Both peptides have the full capacity to stimulate steroidogenesis and cAMP production in bovine adrenal cortical cells.

It is the ultimate aim of this investigation to gain information regarding the chemical nature of ACTH[1] receptors. Conventional approaches to this problem have thus far been unsuccessful; in particular, the receptor appears to lose its affinity for ACTH upon solubilization. We are exploring a combination of affinity labeling and affinity chromatographic techniques based on the avidin–biotin interaction to identify

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[1] Abbreviations: ACTH, adrenocorticotropic hormone; Bct, biocytin; Boc, *tert*-butoxycarbonyl; CCD, countercurrent distribution; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIPEA, *N*-ethyldiisopropylamine; DMF, dimethylformamide; DTBct, dethiobiocytin; HPLC, high-pressure liquid chromatography; OBu[t], *tert*-butyl ester; OSu, *N*-hydroxysuccinimide ester; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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ACTH receptors on adrenocortical cells.

In a previous paper, we have described six photoprobes for ACTH receptors that were prepared by reacting $ACTH_{1-24}$, [Bct^{25}]$ACTH_{1-25}$ amide, and [$DTBct^{25}$]$ACTH_{1-25}$ amide with either 2-nitro-4-azidobenzenesulfenyl chloride or 2-nitro-5-azidobenzenesulfenyl chloride (NAPS). The NAPS derivatives were biologically active and photolysis of adrenal cortical cells in the presence of the 5-NAPS derivatives resulted in covalent attachment, presumably to the ACTH receptors, bringing about persistent activation when the photolyzed cells were incubated in the absence of ACTH (Finn et al., 1985). It should be noted that two of our compounds contain a biotin or dethiobiotin "handle", which should facilitate isolation of photolabeled receptor by the avidin–biotin technique. [Bct^{25}]$ACTH_{1-25}$ amide forms a stable complex with avidin with a $t_{1/2}$ for dissociation of 20 days (Romovacek et al., 1983), and this complex is capable of interacting with ACTH receptors to stimulate steroidogenesis and cAMP production (Finn et al., 1985). Further progress toward ACTH receptor isolation will depend on the availability of radiolabeled biotinylated ACTH derivatives of high specific radioactivities as well as biological activities. Methods to prepare such compounds are described in this paper.

Experimental Procedures

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. H-Glu(OBu^t)-His-Phe-Arg-Trp-GlyOH·HCl, 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. R. Andreatta, Ciba-Geigy Corp., Basel, Switzerland. CMC (carboxymethylcellulose) and Bio-Gel P-2 were from Bio-Rad Corp., Richmond, CA, Amberlite IRA-400 was from Mallinckrodt Chemical Works, St. Louis, MO, di-*tert*-butyl dicarbonate was purchased from Fluka Corp., Buchs, Switzerland, and iodogen was from Pierce Chemical Co., Rockford, IL. Spectra were obtained at the NMR Facilities for Biomedical Studies.

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: (I) 1-butanol–glacial acetic acid–water, 60:20:20; (II) chloroform–methanol–water, 8:3:1 (lower phase); (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 (exposure to hypochlorite followed by spraying the dried plates with a 1:1 mixture of 0.4% KI and 1% starch solution in water), and the biotin reagent *p*-(dimethylamino)cinnamaldehyde (McCormick & Roth, 1970). Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, NY. High-pressure liquid chromatography (HPLC) was performed with a Waters system equipped with a Waters Model 680 automated gradient controller.

NMR Spectra. Spectra were recorded on samples of the materials at approximately 1 mM concentration in ^2H_2O. The pH reported is the uncorrected meter reading obtained on an Orion Model 6100 pH meter. Adjustments of pH were made by addition of microaliquots of ^2HCl or NaO^2H in ^2H_2O. The spectra were obtained on the 600-MHz NMR spectrometer at the NMR Facility for Biomedical Studies, with the FT mode. A total of 1000–2000 scans was accumulated with an acquisition time of 1.36 s and a sweep width of 6024 Hz. A total of 16K transforms was used. The sample temperature was 299 K.

Syntheses. (*A*) *Z-Phe-Ser-Nle-OMe.* N^{α}-Z-Ser-Nle-OMe (Sandrin & Boissonnas, 1963) (2.2 g, 6 mmol) was hydrogenated over Pd for 4 h in MeOH (30 mL) and 10% acetic acid (3.6 mL). The catalyst was removed, the filtrate was evaporated to dryness, and the residue was dried over P_2O_5 and KOH. The dried material was dissolved in DMF (10 mL), Z-Phe-OSu (2.62 g, 6.6 mmol) was added followed by DIPEA (1.03 mL, 6 mmol), and the solution was stirred at room temperature for 40 h. DMF was removed, and the gelatinous residue was dissolved in ethyl acetate (150 mL). The solution was washed in the usual manner, dried over sodium sulfate, and concentrated to a small volume when the product crystallized: yield 1.68 g (55%); mp 154–155 °C; R_f^{I} 0.7, R_f^{II} 0.9, and R_f^{III} 0.8, chlorine-positive, fluorescamine-negative spot; $[\alpha]^{27}_D$ −22.6° (*c* 1.185, MeOH). Anal. Calcd for $C_{27}H_{35}N_3O_7$: C, 63.14; H, 6.87; N, 8.18. Found: C, 63.33; H, 6.69; N, 7.97.

(*B*) *N^{α}-Boc-Ser-Phe-Ser-Nle-OMe.* Z-Phe-Ser-Nle-OMe (1.5 g, 3 mmol) was hydrogenated over Pd for 5 h in MeOH (40 mL) and 10% acetic acid (3.5 mL). The catalyst was removed by filtration, the filtrate was evaporated, and the residue (H-Phe-Ser-Nle-OMe) was dried over P_2O_5 and KOH. *tert*-Butyl nitrite (0.46 mL, 3.96 mmol) was added with stirring to a solution of Boc-Ser-N_2H_3 (723 mg, 3.3 mmol) in DMF (5 mL) and 5.53 N HCl in dioxane (2.4 mL, 13.2 mmol) cooled at −20 °C, and the solution was stirred for 3 min. DIPEA (2.26 mL, 13.2 mmol) was added followed by a solution of H-Phe-Ser-Nle-OMe–acetate in DMF (10 mL) and DIPEA (0.51 mL, 1 mmol). The mixture was stirred at 4 °C for 70 h, the pH being maintained at approximately 8.0 (Hydrion paper) by addition of DIPEA. The solvents were removed in vacuo, the oily residue was dissolved in ethyl acetate (100 mL), and the solution was washed with 1 N citric acid, water, 1 M potassium bicarbonate, and saturated NaCl in the usual manner. The ethyl acetate layers were pooled, dried over sodium sulfate, and concentrated in vacuo with occasional additions of MeOH to prevent precipitation. Ether was added to the semisolid residue, and the mixture was cooled. The gelatinous product was collected, washed with cold ether, and dried: yield 1.36 g (80%); mp 170–172 °C; R_f^{I} 0.8 and R_f^{II} 0.9; $[\alpha]^{27}_D$ −31.66° (*c* 1.289, MeOH). A sample for analysis was recrystallized from acetone–water, mp 175–177 °C. Anal. Calcd for $C_{27}H_{42}N_4O_9$: C, 57.24; H, 7.45; N, 9.89. Found: C, 57.13; H, 7.44; N, 9.91.

(*C*) *N^{α}-Boc-Ser-Phe-Ser-Nle-N_2H_3.* Boc-Ser-Phe-Ser-Nle-OMe (1.25 g, 2.2 mmol) was dissolved in hot MeOH (10 mL), hydrazine hydrate (0.43 mL, 8.8 mmol) was added, and the mixture was allowed to cool. The solid product was collected, washed with cold MeOH, and dried over concentrated sulfuric acid in vacuo. The crude product was recrystallized from boiling 50% aqueous EtOH (30 mL), collected, washed with ice-cold EtOH, and dried: yield 1.09 g (87.2%); mp 212–214 °C (dec); R_f^{I} 0.7 and R_f^{II} 0.4, single spot; amino acid ratios in 24-h acid hydrolysate $Ser_{1.9}$-$Phe_{1.08}$-$Nle_{1.02}$. A sample for analysis was recrystallized from aqueous EtOH. Anal. Calcd for $C_{26}H_{42}N_6O_8 \cdot 0.5H_2O$: C, 54.25; H, 7.53; N, 14.60. Found: C, 54.42; H, 7.64; N, 14.83.

(*D*) *N^{α}-Boc-Ser-Phe-Ser-Nle-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH·HCl. tert*-Butyl nitrite (0.23 mL, 1.94 mmol) was added with stirring to a solution cooled at −20 °C of Boc-Ser-Phe-Ser-Nle-N_2H_3 (997 mg, 1.76 mmol) in DMF (20 mL) containing 5.53 N HCl in dioxane (1.6 mL, 8.82 mmol), and the mixture was stirred at −20 °C for 6 min. DIPEA (1.51 mL, 8.82 mmol) was added followed by a solution of H-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH·HCl (1.36 g, 1.47 mmol) in DMF (40 mL), water (4.5 mL), and DIPEA (0.25

mL, 1.47 mmol). The mixture was stirred for 48 h at 4 °C and was concentrated to approximately 10 mL in vacuo. Aqueous acetic acid (50% v/v, 2 mL) and MeOH (1 mL) were added, and the product was precipitated by addition of ethyl acetate (150 mL). The precipitate was collected, washed with ethyl acetate, and dried, yield 2.38 g. For purification, 500 mg of the crude product was stirred with 0.5% aqueous acetic acid (30 mL) to give a gelatinous mixture that was distributed between 1-butanol (60 mL) and water (30 mL). The precipitate was collected, triturated with water, and dried. The butanol layer was evaporated to a small volume, and additional precipitate was obtained on addition of water. This material was added to the first precipitate: total yield 370 mg (82%); R_f^I 0.4 with trace contaminations. A sample for analysis was dissolved in glacial acetic acid–MeOH (10:1), precipitated with ether, and dried at 100 °C for 12 h. See Table I for amino acid ratios in methanesulfonic acid hydrolysate. Anal. Calcd for $C_{69}H_{96}N_{16}O_{17}\cdot H_2O$: C, 57.57; H, 6.86; N, 15.57. Found: C, 57.39; H, 6.88; N, 15.42.

(*E*) *[Phe²,Nle⁴]ACTH₁₋₂₄*. N^{α}-Boc-Ser-Phe-Ser-Nle-Glu-(OBu^t)-His-Phe-Arg-Trp-Gly-OH (160 mg, 0.11 mmol) was converted to the tosylate (Brundish & Wade, 1973). This tosylate was coupled to H-Lys(Boc)-Pro-Val-Gly-Lys-(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t–dihydrochloride (218 mg, 0.10 mmol) in DMF–pyridine (2:1) (12 mL) containing 1-hydroxybenzotriazole monohydrate (61 mg, 0.40 mmol) with DCC (124 mg, 0.60 mmol) as the condensing reagent (Hofmann et al., 1978). The reaction product was precipitated with ethyl acetate and dried, yield 393 mg. The crude product was purified by CCD (380 transfers) (Schwyzer & Kappeler, 1963), yield 200 mg. This purified peptide was deprotected with 90% TFA containing 2% ethanedithiol (10 mL), and the ion-exchanged product (IRA 400) (158 mg) was purified on CMC in batches of 79 mg each (Allen et al., 1979): total yield 104 mg (36%); R_f^{III} 0.4. See Table I for amino acid ratios in methanesulfonic acid hydrolysate.

(*F*) *H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Bct Amide–Triacetate*. N^{α}-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH–ditosylate prepared from 533 mg (0.24 mmol) of diacetate (Brundish & Wade, 1973) was coupled to biocytin amide–tosylate [prepared from 508 mg (1.18 mmol) of the acetate] in the presence of 1-hydroxybenzotriazole hydrate (216 mg, 1.41 mmol) in DMF–pyridine, 2:1 (15 mL), with DCC (291 mg, 1.14 mmol) as the condensing reagent essentially as described previously (Hofmann et al., 1978) (reaction time 70 h). The trifluoroacetyl group was removed from the reaction product with piperidine (Hofmann et al., 1978), and the partially deprotected and ion-exchanged (IRA 400, acetate cycle) material (534 mg) was purified in two batches on a reverse-phase Sephadex G-50 column (2.4 × 62 cm) (Yamashiro, 1964), which was poured with the lower phase of the system 1-butanol–acetic acid–water (4:1:5) and equilibrated with the upper phase. The product (267 mg) was dissolved in the upper phase (3.5 mL) and applied to the column that was developed with the upper phase: flow rate 0.2 mL/min, fraction size 2 mL. Single-spot material was located in the various fractions by TLC in system I, and these fractions were pooled, evaporated to a small volume, and lyophilized: total yield 290 mg; R_f^I 0.3, single spot. See Table I for amino acid ratios in HCl hydrolysate.

(*G*) *[Phe²,Nle⁴,Bct²⁵]ACTH₁₋₂₅ Amide–Phosphate*. N^{α}-Boc-Ser-Phe-Ser-Nle-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH–tosylate (263 mg, 0.170 mmol) was coupled to H-Lys-(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Bct amide–tritosylate (438 mg, 0.15 mmol) in DMF–pyridine (2:1) (15 mL) containing 1-hydroxybenzotriazole monohydrate (92 mg, 0.60 mmol) with DCC (186 mg, 0.90 mmol) as the condensing reagent. Conditions were similar to those described for the preparation of [Bct²⁵]ACTH$_{1-25}$ amide (Hofmann et al., 1978). The reaction product was purified by CCD (255 transfers) (Schwyzer & Kappeler 1963), and the product was isolated in the usual manner: yield 280 mg; R_f^I 0.3 and R_f^{III} 0.7. The protected peptide (280 mg) was deprotected with 90% TFA containing 2% ethanedithiol (15 mL), and the ion-exchanged product was purified on CMC (Allen et al., 1979): yield 158 mg (32%); R_f^{III} 0.5. See Table I for amino acid ratios in methanesulfonic acid hydrolysate.

(*H*) *[Phe²,Nle⁴,DTBct²⁵]ACTH₁₋₂₅ Amide*. N^{α}-Boc-Ser-Phe-Ser-Nle-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH–tosylate (149 mg, 0.10 mmol) was coupled to H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct amide–tritosylate (Romovacek et al., 1983) (237 mg, 0.082 mmol) in DMF–pyridine (2:1) (8 mL) containing 1-hydroxybenzotriazole monohydrate (50 mg, 0.33 mmol) with DCC (101 mg, 0.49 mmol) as the condensing reagent. Conditions were identical with those described for the preparation of [Bct²⁵]ACTH$_{1-25}$ amide (Hofmann et al., 1978). The reaction product (386 mg) was purified by CCD (250 transfers) (Schwyzer & Kappeler, 1963): yield 130 mg; R_f^I 0.4. The protected peptide was deprotected with 90% TFA containing 2% ethanedithiol (5 mL), and the ion-exchanged product was purified on CMC (Allen et al., 1979): yield 45 mg; R_f^{III} 0.4. See Table I for amino acid ratios in methanesulfonic acid hydrolysate.

Trace Labeling. Iodinations were performed with Iodogen essentially as described by Salacinski et al. (1981). To a microfuge tube coated with iodogen (6.4 μmol) was added 0.05 M pH 7.4 phosphate buffer (30 μL), Na^{125}I (0.5 μmol, 10 μL, 1 μCi), and finally [Phe²,Nle⁴]ACTH$_{1-24}$ (0.6 μmol) in 0.1 mM HCl containing 0.9% NaCl (30 μL), and the mixture was kept at room temperature for 10 min. The reaction was arrested by addition of 0.05 M pH 7.4 phosphate buffer (500 μL). The diluted reaction mixture was kept at room temperature for 15 min. Two 5-μL samples were removed for paper strip chromatography in 10% TCA to determine the degree of ^{125}I incorporation. For separation of free iodine from the peptides, the remaining reaction mixture was applied to a Bio-Gel P-2 column (0.9 × 10 cm) that was equilibrated and eluted with 0.05 M TFA. Aliquots of the solution corresponding to the peptide material were subjected to HPLC on a Bondapak C_{18} column with the solvent system (pump A) 0.05% TFA and (pump B) 0.05% TFA–acetonitrile (1:1). The linear gradient was 40–80% pump B over 20 min with a pumping speed of 2 mL/min. The effluents were monitored by OD at 210 nm and radioactivity. Comparable fractions from several runs were pooled and used for bioassays and spectrophotometry.

Labeling to High Specific Radioactivity. To a 1.5-mL microfuge tube coated with iodogen (16 nmol) was added 0.3 M phosphate buffer, pH 7.4 (45 μL), Na^{125}I (1.6 nmol, 3.5 mCi), and [Phe²,Nle⁴]ACTH$_{1-24}$ (10 nmol) in 0.1 mM HCl containing 0.9% NaCl (30 μL). After 10 min at room temperature, the reaction was arrested by addition of 0.3 M pH 7.4 phosphate buffer (500 μL), and the diluted reaction mixture was kept at room temperature for 15 min. The reaction mixture was applied to a Bio-Gel P-2 column (0.9 ×

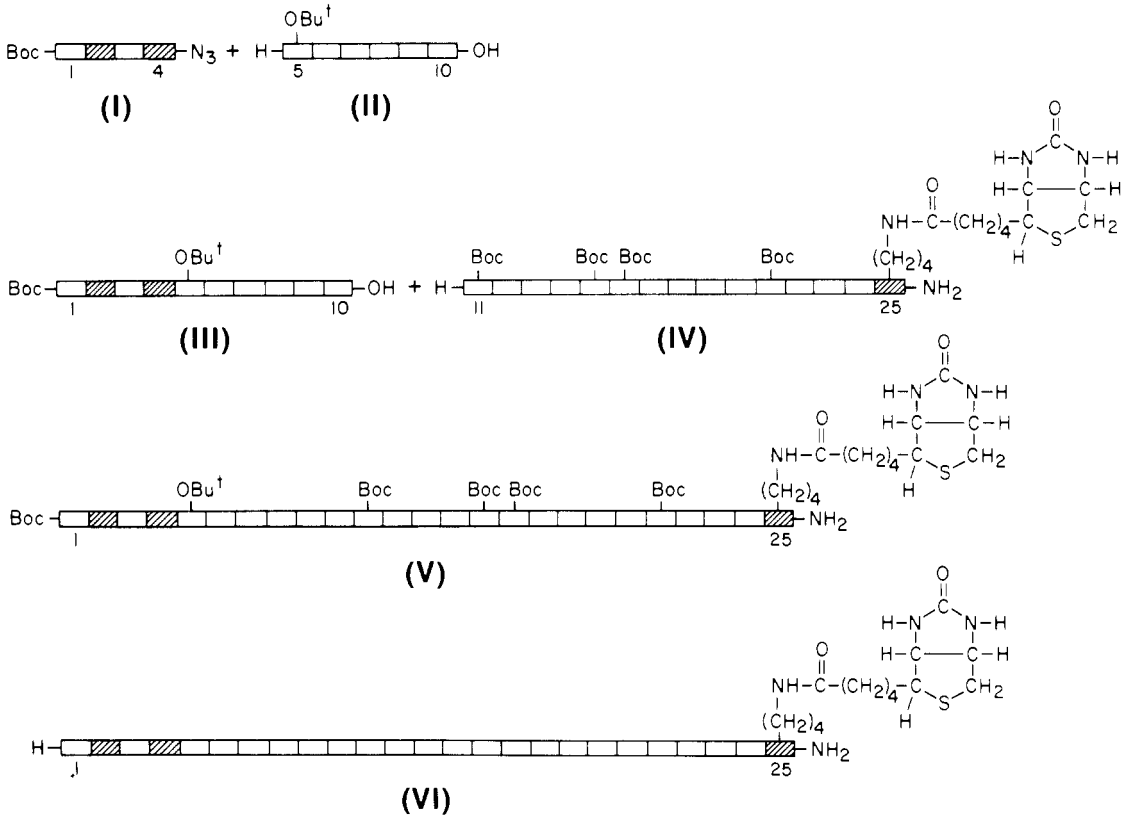

FIGURE 1: Synthetic route to [Phe^2,Nle^4,Bct^{25}]ACTH$_{1-25}$ amide.

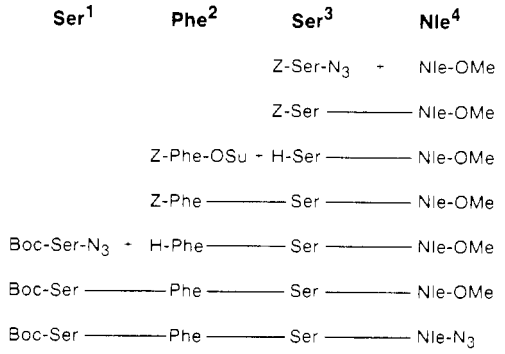

FIGURE 2: Synthetic route to the protected N-terminal fragment.

10 cm), which was equilibrated and eluted with 0.3 M pH 7.4 sodium phosphate buffer. Fractions (0.5 mL each) were collected, and aliquots were counted. The iodinated peptide could be stored at −80 °C for 1 week. Material stored in this manner produced an HPLC pattern that was indistinguishable from that of the freshly labeled peptide.

Bioassays. Stimulation of steroid and cAMP production in calf adrenal cortical cells was determined in the manner previously described (Finn et al., 1985).

Results

The synthetic approach was dictated by the availability, from commercial sources, of two ACTH fragments, i.e., H-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH·HCl (II) and H-Lys-(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t. The strategy for the synthesis of [Phe^2,Nle^4,Bct^{25}]ACTH$_{1-25}$ amide (VI) is illustrated on Figure 1. The azide (I) was prepared in a stepwise manner (Figure 2) and was coupled to II to give the protected decapeptide, III. The synthesis of the pentadecapeptide, IV, containing the biotin residue was performed essentially as described previously (Hofmann et al., 1978), but the method of purification was improved.

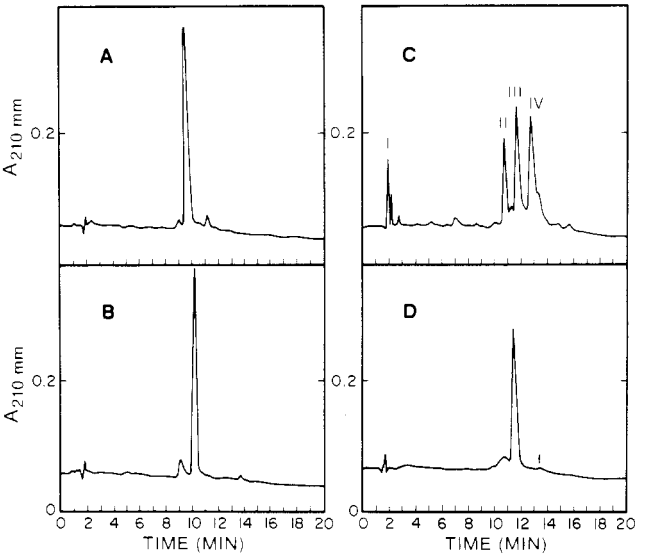

FIGURE 3: HPLC of the following: [Phe^2,Nle^4]ACTH$_{1-24}$ (panel A); [Phe^2,Nle^4,Bct^{25}]ACTH$_{1-25}$ amide (panel B); crude iodination mixture of [Phe^2,Nle^4]ACTH$_{1-24}$ (panel C), (peak I) unknown, (peak II) unreacted [Phe^2,Nle^4]ACTH$_{1-24}$, (peak III) monoiodo-[Tyr^{23}Phe^2,Nle^4]ACTH$_{1-24}$, and (peak IV) diiodo-[Tyr^{23},Phe^2,Nle^4]ACTH$_{1-24}$; [Phe^2,Nle^4,DTBct^2]ACTH$_{1-25}$ amide (panel D). A Bondapak C$_{18}$ column with the following solvent systems was employed: (pump A) 0.05% TFA–(pump B) 50% acetonitrile in 0.05% TFA. The linear gradient was 40–80% pump B over 20 min with a pumping speed of 2 mL/min. Samples of approximately 10 μL were applied.

[Phe^2,Nle^4,Bct^{25}]ACTH$_{1-25}$ amide (VI) was obtained by coupling compound III to compound IV by the DCC–1-hydroxybenzotriazole procedure (König & Geiger, 1970). The protected reaction product (V) was purified by CCD

Table I: Amino Acid Ratios in Acid Hydrolysates of Synthetic ACTH Analogues

amino acid	peptides					
	III	IV	VI	VII	VIII	IX
Trp	0.93 (1)		0.95 (1)	1.05 (1)	1.03 (1)	
Lys		5.01 (5)	5.20 (5)	3.80 (4)	4.76 (5)	5.03 (5)
His	0.82 (1)		0.95 (1)	0.98 (1)	0.86 (1)	
Arg	0.92 (1)	1.91 (2)	3.30 (3)	2.95 (3)	2.89 (3)	2.14 (2)
Ser	2.26 (2)		1.81 (2)	2.10 (2)	2.05 (2)	
Glu	0.84 (1)		1.02 (1)	1.10 (1)	1.03 (1)	
Pro		3.25 (3)	3.19 (3)	3.30 (3)	2.59 (3)	3.17 (3)
Gly	0.93 (1)	0.92 (1)	2.01 (2)	2.20 (2)	2.24 (2)	1.00 (1)
Val		2.96 (3)	2.86 (3)	2.50 (3)	2.97 (3)	2.83 (3)
Nle	1.07 (1)		0.99 (1)	1.05 (1)	0.86 (1)	
Tyr		0.93 (1)	0.99 (1)	0.77 (1)	1.00 (1)	0.83 (1)
Phe	2.03 (2)		1.97 (2)	2.00 (2)	2.11 (2)	

[a] III, Boc-Ser-Phe-Ser-Nle-Glu(Bu^t)-His-Phe-Arg-Trp-Gly-OH; IV, H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Bct amide; VI, [Phe^2,Nle^4,Bct^{25}]ACTH$_{1-25}$ amide; VII, [Phe^2,Nle^4]ACTH$_{1-24}$; VIII, [Phe^2,Nle^4,DTBct^{25}]ACTH$_{1-25}$ amide; IX, H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-DTBct amide. Samples IV and IX were hydrolyzed at 110 °C for 24 h with constant-boiling HCl containing 0.1% phenol. Samples III, VI, VII, and VIII were hydrolyzed with methanesulfonic acid. Figures in parentheses are theoretical values.

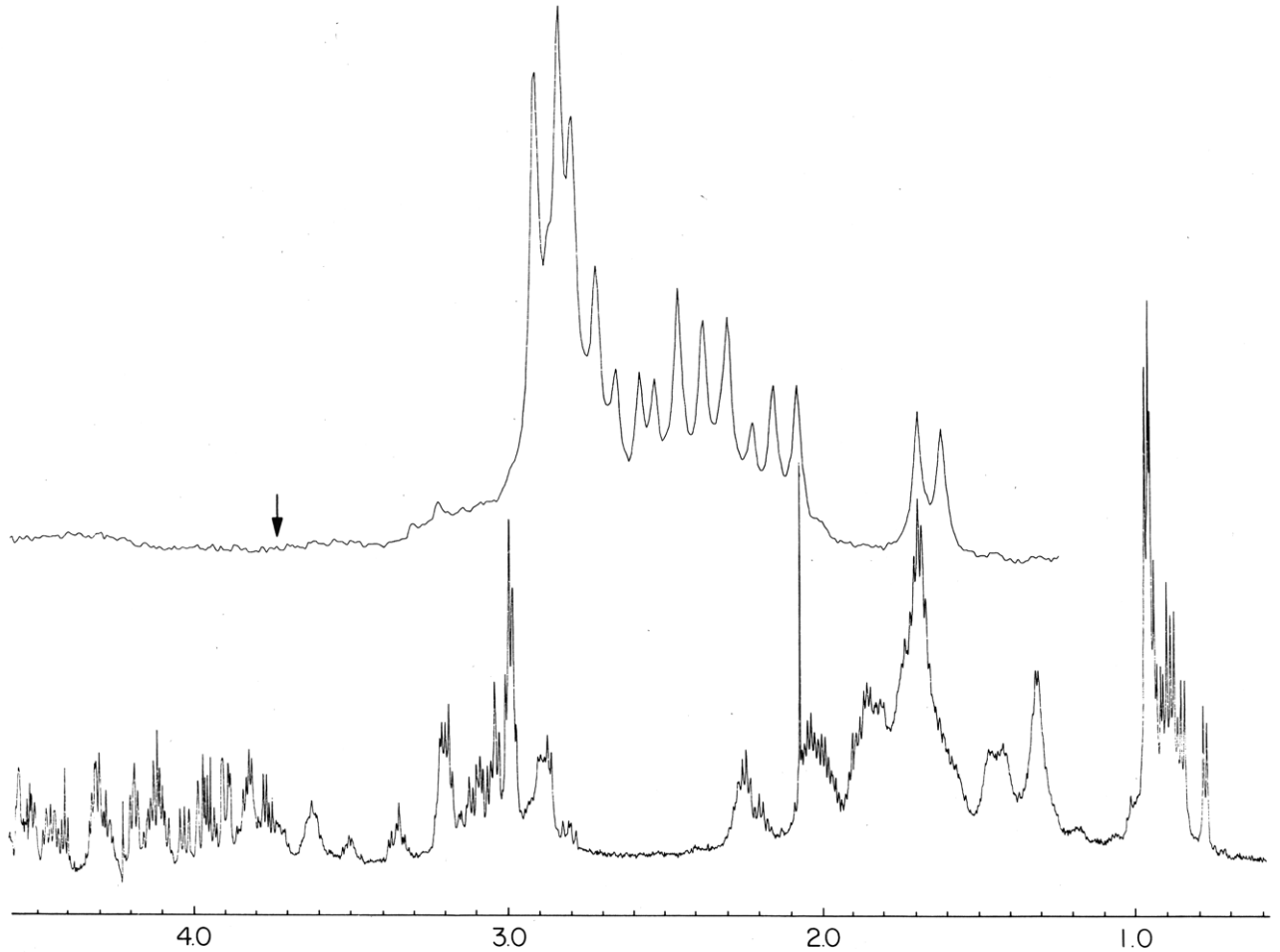

FIGURE 4: Aliphatic region of the 600-MHz FT proton NMR spectrum of [Phe^2,Nle^4]ACTH$_{1-24}$. An expansion of the region from δ 0.7 to δ 1.2 is shown above. The arrow indicates the shift previously observed for the *tert*-butyl group, which is sometimes introduced during the TFA-deprotecting step.

(Schwyzer & Kappeler, 1963). Deprotection with 90% aqueous TFA containing 2% ethanedithiol afforded the final product, which was subjected to ion-exchange chromatography on CMC (Allen et al., 1979). [Phe^2,Nle^4]ACTH$_{1-24}$ (VII) and [Phe^2,Nle^4,DTBct^{25}]ACTH$_{1-25}$ amide (VIII) were synthesized in an analogous manner. The amino acid ratios in acid hydrolysates of the final product and some intermediates are summarized in Table I. The HPLC diagrams of [Phe^2,Nle^4]ACTH$_{1-24}$, [Phe^2,Nle^4,Bct^{25}]ACTH$_{1-25}$ amide, and [Phe^2,Nle^4,DTBct^{25}]ACTH$_{1-25}$ amide are illustrated on Figure 3 (panels A, B, and D).

Exposure of Trp-containing peptides to TFA may result in partial *tert*-butylation of the indole portion of this amino acid (Jaeger et al., 1978). Addition of ethanedithiol to the TFA appears to decrease, but does not eliminate, this side reaction. Using NMR, we have observed that *tert*-butyl groups give a strong, easily observed singlet peak at 1.08 ppm from DSS. The enlarged section of the upfield spectrum, Figure 4, shows that such a peak, if present, is less intense than the noise, which is, in turn, less than $^1/_{30}$ the intensity of the methyl doublet at 0.77 ppm. We conclude that the level of *tert*-butyl introduction was less than 0.5%.

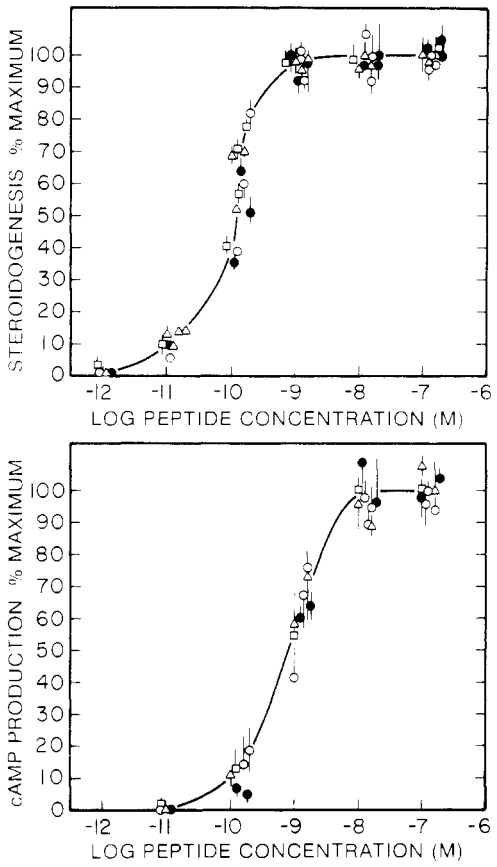

FIGURE 5: Stimulation of steroidogenesis and cAMP production in bovine adrenocortical cells by $ACTH_{1-24}$ (○), [Phe^4,Nle^4]$ACTH_{1-24}$ (●), monoiodo-[Tyr^{23},Phe^2,Nle^4]$ACTH_{1-24}$ (□), and diiodo-[Tyr^{23},Phe^2,Nle^4]$ACTH_{1-24}$ (△).

The stimulation of steroidogenesis and cAMP formation in bovine adrenal cortical cells by [Phe^2,Nle^4]$ACTH_{1-24}$ amide was, within the limits of error of the assay, identical with that of $ACTH_{1-24}$ (Synacthen) (Figure 5). The same was true for [Phe^2,Nle^4,Bct^{25}]$ACTH_{1-25}$ amide and [Phe^2,Nle^4,DTBct]$ACTH_{1-25}$ amide (data not shown).

The optimal conditions for iodination were determined by measuring the effect of varying the reactants individually on the degree of incorporation of radioactivity and on the purity of the product obtained as judged by HPLC. Analysis of the reaction mixtures by paper chromatography in 10% TCA indicated that at iodogen:iodine molar ratios of 1:1, 5:1, and 10:1, 14, 62, and 91% of the radioactivity was incorporated into the [Phe^2,Nle^4]$ACTH_{1-24}$, respectively.

A 10-fold excess of iodogen to iodine was subsequently used to examine the effect of varying the ratio of [Phe^2,Nle^4]-$ACTH_{1-24}$:iodine. The reaction was performed as usual except that the quantity of [Phe^2,Nle^4]$ACTH_{1-24}$ was varied to achieve ACTH:iodine ratios of from 3:1 to 9:1. Unreacted iodine was separated from the peptides by Bio-Gel P-2 chromatography, and the iodinated peptides were examined by HPLC. When the molar ratio of ACTH:iodine was 6:1 or greater, one major radioactive peak, whose retention time on HPLC (see below) corresponded to monoiodo-[Tyr^{23},Phe^2,Nle^4]$ACTH_{1-24}$, accounted for 75% of the radioactivity applied.

In order to have available the monoiodo and diiodo derivatives of [Phe^2,Nle^4]$ACTH_{1-24}$ for biological assays and to determine their retention times on HPLC, we trace labeled a sample of the peptide using a low ratio of [Phe^2,Nle^4]-$ACTH_{1-24}$ to iodine. HPLC of the material, with absorbance at 210 nm for detection, shows the presence of three components with elution times of 9.26 ± 0.79, 10.61 ± 0.68, and 11.83 ± 0.64 min (Figure 3C). When radioactivity served for visualization, the material exhibiting the lowest retention time (peak II) was not detected. Consequently, this material appears to correspond to unchanged starting material, a conclusion that is supported by the finding that its retention time is identical with that of genuine [Phe^2,Nle^4]$ACTH_{1-24}$. The ratio of radioactivity to absorbance at 210 nm provided the information required to identify the two remaining peaks. Specific radioactivity for the material corresponding to peak III isolated by HPLC indicated that this peptide contained 1 atom of iodine per mole of peptide. The material corresponding to peak IV had a ratio of radioactivity to UV absorbance that was twice that of the material corresponding to peak III. On the basis of these findings, we conclude that the material corresponding to peak III is monoiodo-[Phe^2,Nle^4]$ACTH_{1-24}$ and that corresponding to peak IV is diiodo-[Phe^2,Nle^4]$ACTH_{1-24}$. Sufficient material corresponding to peaks III and IV was collected from HPLC runs to determine their steroidogenic and cAMP stimulating activities. As can be seen on Figure 5, the labeled materials are as active as $ACTH_{1-24}$ with respect to both biological activities.

Discussion

This paper is concerned with the development of radioactive probes for ACTH receptors. Ideally, such compounds should retain the full biological activity of the parent hormone while exhibiting high specific radioactivity. Labeling ACTH or ACTH fragments with ^{125}I by the chloramine T or lactoperoxidase procedures results in materials that retain but a small fraction of the binding capacity of the starting materials (McIlhinney & Schulster, 1974; Rae & Schimmer, 1974). The inactivation is the result of methionine oxidation and iodine substitution on Tyr^2 of the ACTH molecule (Lemarie et al., 1977). Geiger et al. (1964) observed that a synthetic analogue of $ACTH_{1-23}$ amide in which Phe replaced Tyr^2 retained biological activity, and it has been found (Hofmann et al., 1964) that the replacement of Met^4 by L-α-amino-*n*-butyric acid in [Gln^5]$ACTH_{1-20}$ amide is compatible with biological activity. It remained for Boissonnas et al. (1966) to establish the equivalence of Met and Nle as concerns the bioactivity of ACTH peptides. Buckley et al. (1981) have reported that an analogue of $ACTH_{1-38}$ in which Tyr^2 is replaced by Phe and Met^4 by Nle can be iodinated with ^{125}I by the chloramine T method without loss of biological activity. Application of this procedure to [Phe^2,Nle^4]$ACTH^{1-24}$ resulted in the formation of a complex mixture of iodinated materials and was not promising. For this reason, we selected the iodogen procedure (Salacinski et al., 1981).

We have prepared [Phe^2,Nle^4]$ACTH_{1-24}$, [Phe^2,Nle^4,Bct^{25}]$ACTH_{1-25}$ amide, and [Phe^2,Nle^4,DT-Bct^{25}]$ACTH_{1-25}$ amide from readily available starting materials by conventional solution methods. HPLC (Figure 3) and amino acid composition of acid hydrolysates (Table I) indicate that the final products are homogeneous. The degree of *tert*-butylation of the Trp residue in the peptides was assessed by NMR (Figure 4) and was found to be less than 0.5%.

In a previous publication, we have described the synthesis of several biotinylated corticotropins (Romovacek et al., 1983). The final step in these syntheses involved acidolytic deprotection of Boc-protected precursors with 90% aqueous TFA containing 2% ethanedithiol for 10 min at ice-bath temperature and 50 min at room temperature. We have now determined the *tert*-butyl content of some of these peptides and of commercially available ACTH (Synacthen) by NMR with the following results: [Bct^{25}]$ACTH_{1-25}$ amide, 2%; [DTBct^{25}]-

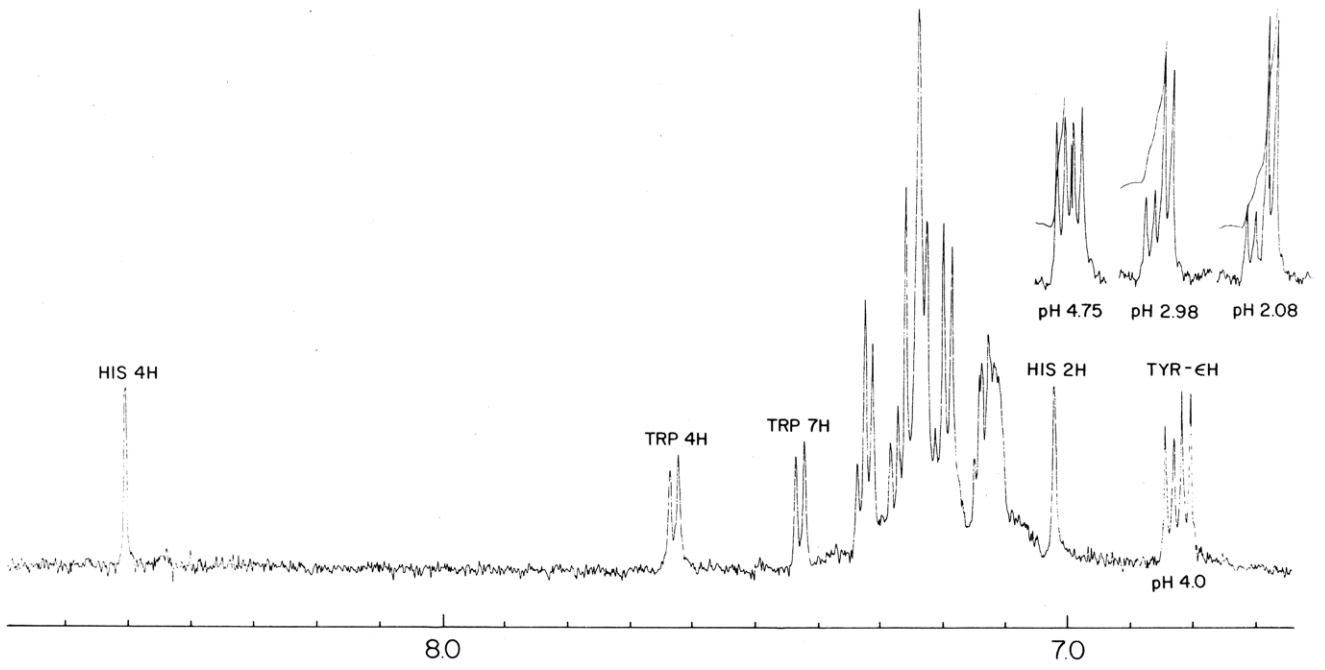

FIGURE 6: Aromatic region of the 600-MHz FT proton NMR spectrum of [Phe^2,Nle^4]ACTH$_{1-24}$. The inserts show the relative intensity variation of the two Tyr^{23} doublets caused by changes in pH and ascribed to cis–trans isomerism of the Tyr–Pro peptide bond.

ACTH$_{1-25}$ amide, 1.5%; Synacthen, 1%. It appears that *tert*-butylation of the Trp residue during acidolytic deprotection of Boc-protected ACTH analogues under the conditions described constitutes a minor side reaction.

Figure 6 displays the aromatic region of the 600-MHz FT proton NMR spectrum of [Phe^2,Nle^4]ACTH$_{1-24}$. The assignments of the signals from the His^6 2- and 4-protons, the Trp^9 4- and 7-protons, and the Tyr^{23} ϵ-protons are trivial and are indicated on the figure. It is interesting that the Tyr ϵ signal is doubled (two sets of doublets) as a consequence of slow cis–trans conversion of the Tyr^{23}–Pro^{24} peptide bond (Deber et al., 1970; Hruby et al., 1971). Observation at several pH values confirmed that this was the cause of the doubling; the relative intensities of the two signals changed, with the downfield doublet contributing nearly 50% at neutral pH and 30% at pH 3.0, in accord with previously reported observations of decreasing cis isomer at low pH (Hetzel & Wüthrich, 1979). The positions of the Trp^9 and Tyr^{23} resonances are not significantly affected by pH changes; however, an upfield shift of one of the aromatic signals from 7.35 to 7.15 appears to occur with a pK_a of approximately 3.5. This must signal a change in environment of either Phe^2 or Phe^7.

The upfield portion of the spectrum is completely in accord with the intended structure. The band of signals centered at 1.30 ppm is absent in ACTH$_{1-24}$ itself and arises from the chain of CH_2 groups of Nle^4. The spectra of the biotinylated and dethiobiotinylated peptides were consistent in each case with the structure expected, being composed of a superposition of the spectrum of the peptide, largely unperturbed by the substitution at the Pro^{24} carboxyl, and of signals appropriate for the substitution, for example, the strong triplet from the α-protons of both the biotin and dethiobiotin, at 2.20 ppm, and the doublet from the methyl group of dethiobiotin in the clear region just downfield from the methyls of the aliphatic amino acids.

The steroidogenic and cAMP stimulating activities of [Phe^2,Nle^4]ACTH$_{1-24}$ and the biocytin and dethiobiocytin analogues of this peptide were, within the limits of the assay procedures, identical with those of the standard ACTH$_{1-24}$ (Figure 5). This is not surprising as substitutions of Tyr^2 by Phe or Met^4 by Nle are, as has been pointed out, compatible with biological activity. Furthermore, we have previously shown that attaching biocytin or dethiobiocytin to the carboxy-terminal amino acid does not alter biological activity (Romovacek et al., 1983). The finding (Figure 5) that 1 or 2 atoms of iodine can be introduced into [Phe^2,Nle^4]ACTH$_{1-24}$ without detectable loss of biological activity makes this molecule and its biocytin and dethiobiocytin analogues attractive probes for detecting ACTH receptors.[2]

The combined use of iodogen and [Phe^2,Nle^4]ACTH$_{1-24}$ in excess over ^{125}I permits almost quantiative incorporation of radioactivity (sp act. 0.4 mCi/nmol) and results in the introduction of 75% of the radioactivity into a single product whose retention time on HPLC corresponds to that of the monoiodo derivative.

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[2] Since submission of the manuscript, Gallo-Payet and Escher (1985) have shown that ^{125}I-[Phe^2,Nle^4]ACTH$_{1-24}$ binds specifically to zona fasciculata cells from rat adrenals.

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Evidence for the Existence of a Channel in the Glucose-Specific Carrier EII^{Glc} of the *Salmonella typhimurium* Phosphoenolpyruvate-Dependent Phosphotransferase System†

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ABSTRACT: The effect of membrane-impermeable sulfhydryl reagents on glucose-specific enzyme II (EII^{Glc}) activity has been studied in *Salmonella typhimurium* whole cells and in properly sealed inverted cytoplasmic membrane vesicles. Glutathione *N*-hexylmaleimide and *N*-polymethylenecarboxymaleimides inactivate methyl α-D-glucopyranoside (α-MeGlc) transport and phosphorylation in whole cell preparations at a dithiol that can be protected by oxidizing reagents, trivalent arsenicals, or phosphorylation of EII^{Glc}. Accessibility to this activity-linked site is restricted to small apolar reagents or to polar reagents with a hydrophobic spacer between the polar group and the reactive maleimide moiety. These same reagents inactivate α-MeGlc phosphorylation in inverted cytoplasmic membrane vesicles. Inhibition results from reaction at a dithiol that can be protected by nonpermeant mercurials, oxidants, and arsenicals as well as by phosphorylation of EII. The characteristics of this site are virtually identical with those of the activity-linked dithiol elucidated in intact cells. No evidence could be found for a second activity-linked site on the other side of the membrane when the permeable reagent *N*-ethylmaleimide was used. Since only one activity-linked dithiol can be detected with sealed inverted membrane vesicles or intact cells and it is accessible to membrane-impermeable sulfhydryl reagents from both sides of the cytoplasmic membrane, we suggest that it is located in a channel constructured by the carrier and that the channel spans the membrane. A second dithiol, not essential for activity, is located near the outer surface of the cytoplasmic membrane.

The phosphoenolpyruvate-dependent methyl α-D-glucopyranoside (α-MeGlc)[1] transport activity in *Escherichia coli* and *Salmonella typhimurium* whole cells and membrane vesicles responds to two energy sources, phosphoenolpyruvate and a $\Delta\mu_{H^+}$. Phosphoenolpyruvate (PEP) drives α-MeGlc accumulation via a $P\text{–}EII^{Glc}$ intermediate (Misset et al., 1983; Peri et al., 1984; Begley et al., 1982). The $\Delta\mu_{H^+}$ inhibits α-MeGlc transport (Reider et al., 1979, Hernandez-Asensio et al., 1975; Del Campo et al., 1975; Robillard & Konings, 1981). Previous studies demonstrated that phosphorylation alters the ability of sulfhydryl reagents to inactivate EII^{Glc}

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[1] Abbreviations: PEP, phosphenolpyruvate; EII^{Glc}, glucose-specific enzyme II of the PEP-dependent phosphotransferase system; EII^{Mtl}, mannitol-specific enzyme II; GSM, glutathione *N*-hexylmaleimide; α-MeGlc, methyl α-D-glucopyranoside; DTT, dithiothreitol; AM2, -3, -5, and -10, *N*-polymethylenecarboxymaleimides containing 2, 3, 5, or 10 methylene moieties between the carboxylate group and the maleimide ring; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; PCMBS, *p*-(chloromercuri)benzenesulfonate; DNP, 2,4-dinitrophenol; thorin, 4-[(2-arsonophenyl)azo]-3-hydroxy-2,7-naphthalenedisulfonic acid; BAL, British antilewesite (2,3-dimercaptopropanol); Tris, tris(hydroxymethyl)aminomethane; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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