# Synthesis of Corticotropin Peptides. XVI. The Total Syntheses of Porcine and Human Corticotropins*

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The syntheses are described of two nonatriacontapeptides corresponding to the primary structure of porcine corticotropin ($\alpha_p$-ACTH) and that of human hormone ($\alpha_h$-ACTH). The porcine peptide is synthesized by assembling three fragment peptides corresponding to ACTH(1—10), ACTH(11—21) and $\alpha_p$-ACTH (22—39). The Synthetic product is compared with natural $\alpha_p$-ACTH in terms of chemical, physicochemical, and biological properties to establish their identity. The human peptide is synthesized *via* two different routes, one of which is the same as that employed for the synthesis of porcine peptide. The two synthetic preparations of human peptide are not only indistinguishable from each other, but also very similar to $\alpha_p$-ACTH in various items of physicochemical measurements and in the *in vivo* steroidogenesis assay. The collected data prove the satisfactory synthesis of $\alpha_h$-ACTH as well as $\alpha_p$-ACTH.

The thirty-nine amino acid sequence of porcine corticotropin ($\alpha_p$-ACTH) was first proposed by Shepherd *et al.*[1] in 1956 and its total synthesis was accomplished by Schwyzer and Sieber[2] in 1963. The primary structure tentatively proposed for the human hormone ($\alpha_h$-ACTH)[3] was synthesized by Bajusz *et al.*[4] in 1967.

Natural $\alpha_p$-ACTH was known to be deamidated upon mild alkaline treatment.[5] Recently, it has also been found by Gráf *et al.*[6] and Riniker *et al.*[7] that the natural preparations of $\alpha_p$-ACTH and $\alpha_h$-ACTH undergo deamidation at the same rate in dilute aqueous ammonia. However, the synthetic product of $\alpha_p$-ACTH[2,7] and that of $\alpha_h$-ACTH[4,6] were stable under the same conditions. These synthetic peptides contained a single amide group in the form of Gln 30. The inconsistency observed between the natural and the synthetic preparations prompted them to reexamine the structures of these two mammalian hormones. As a result, the primary structure of $\alpha_p$-ACTH was revised in 1971.[6,7] At the same time, the correct amino acid sequence 22—39 (a tryptic fragment) of $\alpha_h$-ACTH was also presented.[7] Subsequent to the above revisions, the 1—20 sequence of $\alpha_h$-ACTH, which had been proposed only on the homology with the hormones from other species, was confirmed by Bennett *et al.*[8] Figure 1 shows the amino acid sequences of porcine and human hormones thus established; note that the only structural difference between the two mammalian hormones resides in position 31, Leu in $\alpha_p$-ACTH and Ser in $\alpha_h$-ACTH, and that these structures contain a single amide group in the form of Asn 25. The synthesis of the revised structure of $\alpha_h$-ACTH was reported by Sieber *et al.*[9] Kisfaludy *et al.*[10] and Nishimura *et al.*[11] A solid-phase synthesis was described by Yamashiro and Li.[12] Very recently Yajima *et al.* have accomplished the synthesis of $\alpha_p$-ACTH.[13] We wish now to report the total syntheses of $\alpha_p$-ACTH and $\alpha_h$-ACTH performed on the basis of our own strategy.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| $\alpha_p$-ACTH (Ip): | H-Ser- | Tyr- | Ser- | Met- | Glu- | His- | Phe- | Arg- |
| $\alpha_h$-ACTH (Ih): | H-Ser- | Tyr- | Ser- | Met- | Glu- | His- | Phe- | Arg- |

| | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|
| (Ip): | Trp- | Gly- | Lys- | Pro- | Val- | Gly- | Lys- | Lys- | Arg— |
| (Ih): | Trp- | Gly- | Lys- | Pro- | Val- | Gly- | Lys- | Lys- | Arg— |

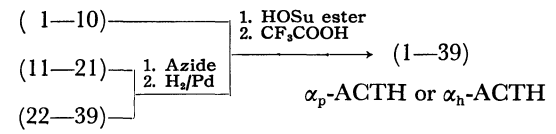
| | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|
| (Ip): | Arg- | Pro- | Val- | Lys- | Val- | Tyr- | Pro- | Asn- | Gly— |
| (Ih): | Arg- | Pro- | Val- | Lys- | Val- | Tyr- | Pro- | Asn- | Gly— |

| | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
|---|---|---|---|---|---|---|---|---|---|
| (Ip): | Ala- | Glu- | Asp- | Glu- | **Leu**- | Ala- | Glu- | Ala- | Phe- |
| (Ih): | Ala- | Glu- | Asp- | Glu- | **Ser**- | Ala- | Glu- | Ala- | Phe- |

| | 36 | 37 | 38 | 39 |
|---|---|---|---|---|
| (Ip): | Pro- | Leu- | Glu- | Phe-OH |
| (Ih): | Pro- | Leu- | Glu- | Phe-OH |

Fig. 1. Primary structures of porcine corticotropin ($\alpha_p$-ACTH)[1,6,7] and human corticotropin ($\alpha_h$-ACTH).[3,7,8]

In the preceding papers, we described the synthesis of an undecapeptide derivative corresponding to ACTH-(11—21),[14] and that of octadecapeptides, $\alpha_p$-ACTH-(22—39) and $\alpha_h$-ACTH(22—39).[15] The two octadecapeptides were obtained simultaneously with an identical synthetic procedure, except for the introduction of the amino acid residue in position 31. The fully deblocked porcine peptide was identified with the $\alpha_p$-ACTH(22—39) isolated from a tryptict digest of



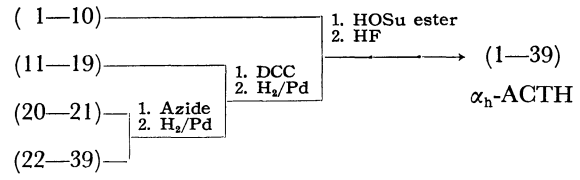

( 1—10): Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-OH (II)[16]

(11—21): Z-Lys(Mhoc)-Pro-Val-Gly-Lys(Mhoc)-Lys(Mhoc)-Arg-Arg-Pro-Val-Lys(Mhoc)-$NHNH_2$ (III)[14]

(22—39): H-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu$^t$)-Asp(OBu$^t$)-Glu(OBu$^t$)-R-Ala-Glu(OBu$^t$)-Ala-Phe-Pro-Leu-Glu(OBu$^t$)-Phe-OBu$^t$ (IVp: R=Leu, IVh: R=Ser)[15]

Fig. 2. Outlined route for the synthesis of $\alpha_p$-ACTH and $\alpha_h$-ACTH (route A). Z: benzyloxycarbonyl, Boc: *t*-butoxycarbonyl, Bu$^t$: *t*-butyl, Mhoc: 1-methylcyclohexyloxycarbonyl, HOSu: *N*-hydroxysuccinimide.

* All the amino acid residues mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature [*Biochemistry*, **5**, 2585 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972)], and include Mhoc: 1-methylcyclohexyloxycarbonyl.




(11—19): Z-Lys(Mhoc)-Pro-Val-Gly-Lys(Mhoc)-Lys(Mhoc)-Arg($NO_2$)-Arg($NO_2$)-Pro-OH (V)[14]
(20—21): Z-Val-Lys(Mhoc)-$NHNH_2$[19c]

Fig. 3. Alternative synthesis of $\alpha_h$-ACTH (route B). Z: benzyloxycarbonyl, Mhoc: 1-methylcyclohexyloxycarbonyl, HOSu: *N*-hydroxysuccinimide, DCC: dicyclohexylcarbodiimide.
For (1—10) and (22—39), see Fig. 2.

natural $\alpha_p$-ACTH.[15] This proved the satisfactory synthesis of porcine peptide and that of human peptide as well. The synthesis of the decapeptide derivative corresponding to ACTH(1—10) was reported previously.[16] The present paper describes the formation of the nonatriacontapeptides, corresponding to $\alpha_p$-ACTH and $\alpha_h$-ACTH, from these fragment peptides according to the route illustrated in Fig. 2. An alternative synthesis of $\alpha_h$-ACTH is also described, in which a nonapeptide derivative corresponding to ACTH(11—19)[14] is utilized as an intermediate (Fig. 3).

*Synthesis of Porcine Hormone, $\alpha_p$-ACTH (Ip).* The synthesis started with the coupling of the C-terminal octadecapeptide derivative (IVp, Fig. 2) and the undecapeptide azide, derived from III (Fig. 2) by the treatment with an alkyl nitrite in an anhydrous acid solution,[17] to give a nonacosapeptide Z-Lys(Mhoc)-Pro-Val-Gly-Lys(Mhoc)-Lys(Mhoc)-Arg-Arg-Pro-Val-Lys(Mhoc)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu$^t$)-Asp(OBu$^t$)-Glu(OBu$^t$)-Leu-Ala-Glu(OBu$^t$)-Ala-Phe-Pro-Leu-Glu(OBu$^t$)-Phe-OBu$^t$ (IXp). The crude preparation of IXp was purified by partition chromatography on a column of Sephadex LH-20 with 1-butanol-acetic acid-water (4: 1: 2) as solvent. The sufficient purity was confirmed by TLC and by amino acid analysis of the acid hydrolysate. The yield was 64%. Compound IXp thus obtained was then submitted to catalytic hydrogenolysis and the product was chromatographed on a silica gel column with ethyl acetate-acetic acid-water (4: 1: 1) as solvent to give the $N^{\alpha}$-free nonacosapeptide (Xp) corresponding to $\alpha_p$-ACTH-(11—39).

The protected decapeptide (II, Fig. 2) corresponding to ACTH(1—10) was synthesized as reported previously,[16] except that in the azide couplings involved Boc-Ser-Tyr-$NHNH_2$ and Boc-Ser-Tyr-Ser-Met-$NHNH_2$ were converted into the corresponding azides by the treatment with an alkyl nitrite,[17] instead of nitrous acid formerly used, to improve the coupling yields significantly. The crude preparation of II was purified on a silica gel column with ethyl acetate-acetic acid-water (4: 1: 1) as solvent. The final reaction connecting the N-terminal decapeptide with the rest of the molecule of $\alpha_p$-ACTH was performed by the *N*-hydroxysuccinimide (HOSu) ester method.[18] The same procedure had been employed in our synthesis of the ACTH peptides with shorter chain lengths.[19] The decapeptide active ester derived from II was allowed to react with Xp obtained above to give a protected nonatriacontapeptide Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys(Mhoc)-Pro-Val-Gly-Lys(Mhoc)-Lys(Mhoc)-Arg-Arg-Pro-Val-Lys(Mhoc)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu$^t$)-Asp(OBu$^t$)-Glu(OBu$^t$)-Leu-Ala-Glu(OBu$^t$)-Ala-Phe-Pro-Leu-Glu(OBu$^t$)-Phe-OBu$^t$. The crude product was, without purification, deprotected with trifluoroacetic acid in the presence of anisole and 2-mercaptoethanol as scavengers, followed by the treatment with Amberlite CG-400 (acetate form). Purification of the product was achieved by repeated chromatography on a carboxymethyl cellulose column using an ammonium acetate buffer with a linear concentration gradient of 0—0.4 M (Figs. 4 and 5). The synthetic $\alpha_p$-ACTH (Ip) thus obtained was found to be homogeneous in TLC and its acid hydrolysate contained the constituent amino acids in the ratios predicted by theory with an exception of tryptophan. The Tyr/Trp ratio in intact Ip was 2.06,

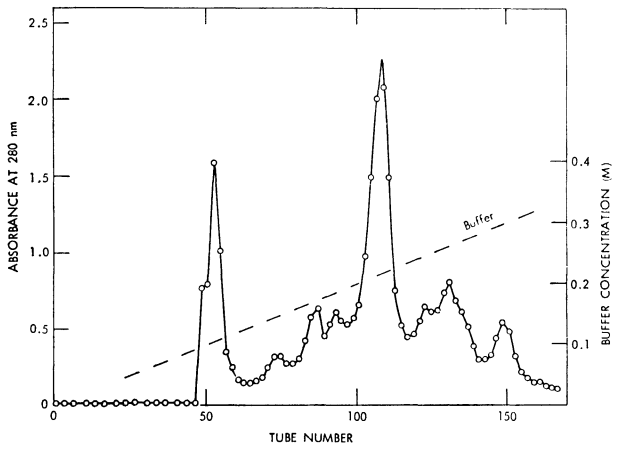



Fig. 4. Carboxymethyl cellulose column chromatography of crude synthetic $\alpha_p$-ACTH: sample, 0.46 g; column, CM-52 (Whatman), 2.1×22 cm; buffer, 0—0.4 M ammonium acetate (pH 6.5), 1800 ml; fractionation, 9 ml/tube.

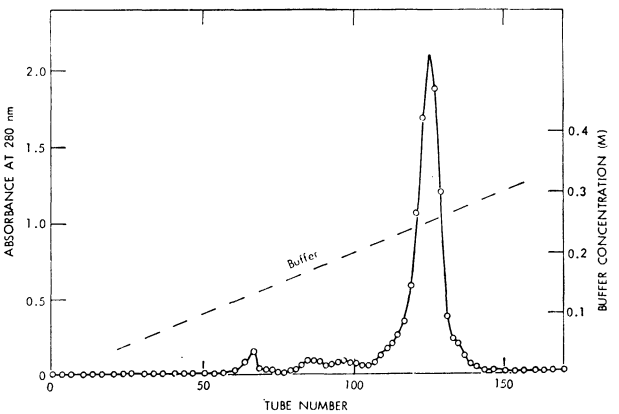



Fig. 5. Rechromatography of partially purified synthetic $\alpha_p$-ACTH on a carboxymethyl cellulose column: sample, 0.15 g, derived from Fig. 4 (for details see Experimental); column, CM-52 (Whatman), 2.8×22 cm; buffer, 0—0.4 M ammonium acetate (pH 6.5), 1800 ml; fractionation, 9 ml/tube.

as determined spectrophotometrically.[20] The overall yield of Ip for the final coupling, deprotection and purification processes was 26%, when the molecular weight of 5050, estimated from ultraviolet absorption on the assumption that $E^{1\%}_{1cm}=17.7$ (at $\lambda^{0.1MHCl}_{max}=276$ nm) for 100% peptide content,[9] was employed.

An authentic sample of $\alpha_p$-ACTH to be compared with the synthetic peptide was obtained by purification of a commercial preparation from porcine origin. The crude preparation (Sigma)[21] was submitted to chromatography on a column of carboxymethyl cellulose, in which 0.1 M sodium acetate (pH 6.2) was used as a buffer and the elution of peptide was effected by increasing the concentration of sodium chloride added to the buffer. The fractions containing the desired material were combined and passed through an Amberlite XAD-2 column to remove inorganic salts. The peptide remained on the column was eluted with a mixture of ethanol–0.1 M acetic acid (1: 1) quantitatively. The purified porcine hormone was found to be homogeneous in TLC and amino acid analysis also confirmed its sufficient purity. The Tyr/Trp ratio in intact peptide was 1.94, as determined spectrophotometrically.[20]

The synthetic peptide (Ip) and the natural hormone, obtained as described above, were indistinguishable from each other by electrophoresis on polyacrylamide gel at pH 4.0 (Fig. 6)[22] and pH 9.4 (Fig. 7)[23] and by TLC (cellulose) with 1-butanol–acetic acid–pyridine–water (30: 6: 20: 24) as solvent. The ultraviolet absorption spectra of the synthetic and natural preparations were identical within the precision of measurements. The optical rotatory dispersion and circular dichroism spectra of the synthetic peptide were virtually identical with those of the natural hormone as shown in Tables 1 and 2.[24]

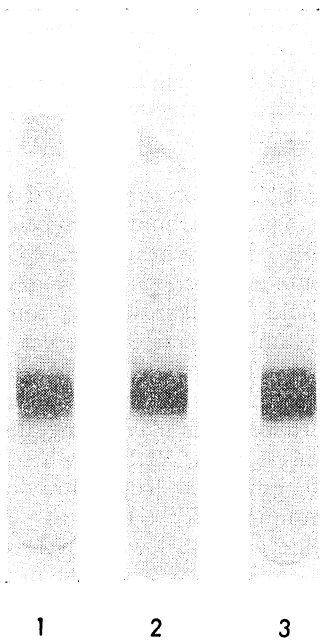



Fig. 6. Electrophoresis of $\alpha_p$-ACTH and $\alpha_h$-ACTH preparations on 15% polyacrylamide gel at pH 4.0: 1, natural $\alpha_p$-ACTH, 60 μg; 2, synthetic $\alpha_p$-ACTH, 60 μg; 3, synthetic $\alpha_h$-ACTH, 60 μg. Detection, by staining with Amido-Black 10B.

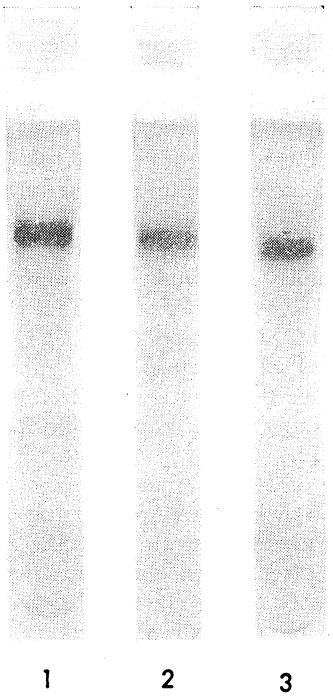



Fig. 7. Electrophoresis of $\alpha_p$-ACTH and $\alpha_h$-ACTH preparations on 7.5% polyacrylamide gel at pH 9.4: 1, natural $\alpha_p$-ACTH, 20 μg; 2, synthetic $\alpha_p$-ACTH, 20 μg; 3, synthetic $\alpha_h$-ACTH, 20 μg. Detection, by staining with Amido-Black 10B.

Table 1. Optical rotatory dispersion of natural and synthetic corticotropins[24]

| λ (nm) | $[\alpha]^{25}_{\lambda}$ | | |
|---|---|---|---|
| | Natural $\alpha_p$-ACTH | Synthetic $\alpha_p$-ACTH | Synthetic $\alpha_h$-ACTH |
| 450 | −211 | −216 | −219 |
| 400 | −309 | −313 | −296 |
| 350 | −407 | −433 | −389 |
| 300 | −722 | −753 | −692 |
| 250 | −1660 | −1760 | −1560 |
| 240 | −2160 | −2330 | −1940 |
| 230 | −2920 | −3050 | −2790 |

Table 2. Circular dichroism of natural and synthetic corticotropins[24]

| λ (nm) | $[\theta]^{25}_{\lambda}$ | | |
|---|---|---|---|
| | Natural $\alpha_p$-ACTH | Synthetic $\alpha_p$-ACTH | Synthetic $\alpha_h$-ACTH |
| 310 | 0 | 0 | 0 |
| 287 | −37 | −43 | −33 |
| 260 | 0 | 0 | 0 |
| 239 | −280 | −283 | −210 |
| 233 | — | — | 0 |
| 232 | 0 | 0 | — |

Electrophoretic patterns of a tryptic hydrolysate of the synthetic material were identical with those obtained with the natural hormone. The tryptic digestion

[substrate: enzyme=50 (w/w)] was carried out at pH 8.2 and 37 °C for 60 min and the electrophoresis on a cellulose plate (Cellulose F, Merck) was performed at pH 6.9 in 0.1 M collidine acetate. Recently, it has been shown that natural $\alpha_p$-ACTH undergoes alkaline deamidation at the asparagine residue in position 25 to form the corresponding aspartyl-$\alpha/\beta$-peptide[6,7] which can be distinguished from the intact hormone by electrophoresis on polyacrylamide gel at pH 9.[6] This was equally observed with our synthetic and natural preparations of porcine ACTH, when they were treated with 0.1 M ammonia at 37 °C according to Gráf *et al.*[6] Their electrophoretic patterns are not shown here, since they were virtually the same as those obtained with our synthetic human hormone which are shown in Fig. 12.

Finally, the synthetic material and the authentic porcine hormone were compared for the *in vivo* steroidogenic activity to demonstrate that the former was as active as the latter, when the assays were performed by a method based on the elevated levels of 11-hydroxycorticosteroids in the adrenal venous blood of the hypophysectomized rat in response to intravenous administration of a test sample (Table 3).[25]

TABLE 3. *In vivo* STEROIDOGENIC ACTIVITY OF NATURAL AND SYNTHETIC CORTICOTROPINS[25]

| Peptide | | Activity[a] (units/mg) |
|---|---|---|
| $\alpha_p$-ACTH | Natural | 94.2 |
| | Synthetic | 98.1 |
| $\alpha_h$-ACTH | Synthetic (route A)[b] | 98.6 |
| | Synthetic (route B)[c] | 98.7 |

a) The Third USP Corticotropin Reference Standard was used as reference. b) See Fig. 2. c) See Fig. 3.

In view of the correspondence between our synthetic $\alpha_p$-ACTH and the natural hormone in terms of chemical, physicochemical and biological properties, we conclude that their identity has now been established.

*Synthesis of Human Hormone, $\alpha_p$-ACTH (Ih).* The human hormone was synthesized by two different routes A (Fig. 2) and B (Fig. 3). Route A is exactly the same as that employed for the synthesis of the porcine hormone, except that the octadecapeptide derivative (IVh, Fig. 2) was used as a starting material. The intermediate nonacosapeptide, Z-Lys(Mhoc)-Pro-Val-Gly-Lys(Mhoc)-Lys(Mhoc)-Arg-Arg-Pro-Val-Lys(Mhoc)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu$^t$)-Asp(OBu$^t$)-Glu(OBu$^t$)-Ser-Ala-Glu(OBu$^t$)-Ala-Phe-Pro-Leu-Glu(OBu$^t$)-Phe-OBu$^t$ (IXh), corresponding to $\alpha_h$-ACTH(11—39) was obtained in a 63% yield after purification on a Sephadex LH-20 column. Catalytic hydrogenolysis of IXh yielded the $N^\alpha$-free peptide, which was coupled with the decapeptide (II) by the HOSu ester method[18] to give a protected nonatriacontapeptide Boc-Ser-Tyr-Ser-Met-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys(Mhoc)-Pro-Val-Gly-Lys(Mhoc)-Lys(Mhoc)-Arg-Arg-Pro-Val-Lys(Mhoc)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu$^t$)-Asp(OBu$^t$)-Glu(OBu$^t$)-Ser-Ala-Glu(OBu$^t$)-Ala-Phe-Pro-Leu-Glu(OBu$^t$)-Phe-OBu$^t$. The crude product was deblocked with trifluoroacetic acid

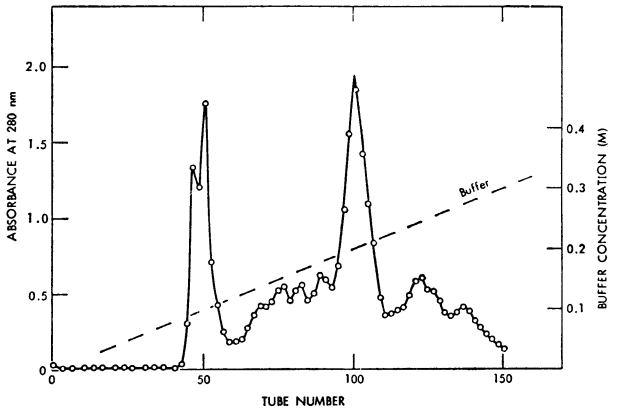



Fig. 8. Carboxymethyl cellulose column chromatography of crude $\alpha_h$-ACTH synthesized *via* route A: sample, 0.45 g; column, CM-52 (Whatman), 2.1×22 cm; buffer, 0—0.4 M ammonium acetate (pH 6.5), 1800 ml; fractionation, 9 ml/tube.

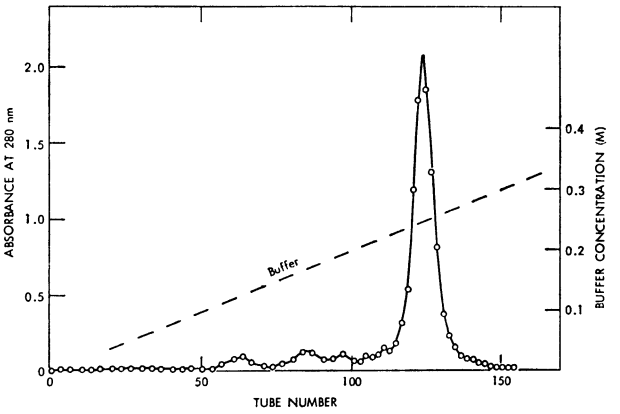



Fig. 9. Rechromatography of partially purified synthetic $\alpha_h$-ACTH on a carboxymethyl cellulose column: sample, 0.13 g, derived from Fig. 8 (for details see Experimental); column, CM-52 (Whatman), 2.8×22 cm; buffer, 0—0.4 M ammonium acetate (pH 6.5), 1800 ml; fractionation, 9 ml/tube.

and the resulting free peptide isolated in the form of acetate was purified on carboxymethyl cellulose columns (Figs, 8 and 9). The synthetic $\alpha_h$-ACTH (Ih) thus obtained by route A behaved as a single component in TLC and its acid hydrolysate was found to contain the constituent amino acids in correct ratios with an exception of tryptophan. The Tyr/Trp ratio in intact Ih was 1.98, as determined spectrophotometrically,[20] The overall yield of Ih for the final coupling, deprotection and purification processes was 21.5%, when the molecular weight of 4960, estimated from ultraviolet absorption on the assumption that $E^{1\%}_{1cm}=17.7$ (at $\lambda^{0.1MHCl}_{max}=276$ nm) for 100% peptide content,[9] was employed.

Route B, shown in Fig. 3, represents an alternative synthesis of $\alpha_h$-ACTH. First, the $N^\alpha$-free octadecapeptide (IVh, Fig. 2) was acylated with Z-Val-Lys(Mhoc)-$N_3$, derived from the corresponding hydrazide (Fig. 3)[19c] by the treatment with nitrous acid in the usual manner, to give a protected eicosapeptide (VI) in a 91% yield. Compound VI was submitted to catalytic hydrogenolysis and the resulting $N^\alpha$-free eicosapeptide was coupled

with nonapeptide V (Fig. 3) corresponding to ACTH-(11—19) by means of DCC in the presence of 1-hydroxybenzotriazole.[26] The product was purified on a silica gel column with chloroform–methanol (9: 1) as solvent to afford a nonacosapeptide, Z–Lys(Mhoc)–Pro–Val–Gly–Lys (Mhoc) – Lys (Mhoc) – Arg ($NO_2$) – Arg ($NO_2$)–Pro–Val–Lys (Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu-($OBu^t$)–Asp ($OBu^t$) –Glu ($OBu^t$) –Ser–Ala–Glu ($OBu^t$)–Ala–Phe–Pro–Leu–Glu($OBu^t$)–Phe–$OBu^t$ (VII), in a 47% yield. Simultaneous removal of the benzyloxycarbonyl (Z) and two nitro groups from VII was attempted by catalytic hydrogenolysis. However, the reaction gave a product which was reactive to ninhydrin but not to the Sakaguchi reagent. The product was also found to have a maximum absorption at 270 nm ($E^{1\%}_{1cm}$=62.3). These observations strongly suggest that the nitro groups had remained almost intact even after extensive hydrogenolysis. The $N^{\alpha}$-free nonacosapeptide (VIII) thus obtained was then allowed to react with the HOSu ester of decapeptide II (Fig. 2) to give a protected nonatriacontapeptide Boc–Ser–Tyr–Ser–Met–Glu-($OBu^t$)–His–Phe–Arg–Trp–Gly–Lys (Mhoc)–Pro–Val–Gly–Lys (Mhoc) – Lys (Mhoc) – Arg ($NO_2$) – Arg ($NO_2$) – Pro–Val–Lys (Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu-($OBu^t$) – Asp($OBu^t$) – Glu($OBu^t$) – Ser–Ala – Glu($OBu^t$)–Ala–Phe–Pro–Leu–Glu($OBu^t$)–Phe–$OBu^t$. The crude product was deprotected by the hydrogen fluoride treatment in the presence of anisole as scavenger[27] to liberate $\alpha_h$-ACTH as the hydrofluoride. This was treated with Amberlite CG-400 (acetate) in the usual manner. The crude material was purified by chromatography on a carboxymethyl cellulose column using an ammonium acetate buffer (Figs. 10 and 11). The purity of $\alpha_h$-ACTH (Ih) thus synthesized by route B was confirmed by TLC and by amino acid analysis. The overall yield of Ih for the final coupling, deprotection and purification processes was 29%, as based on the molecular weight of 4960 which was determined spectrophotometrically.

The two synthetic preparations of $\alpha_h$-ACTH obtained above were indistinguishable from each other by chromatographic and electrophoretic behavior. Their ultraviolet absorption spectra and optical rotations were virtually identical. In addition, they exhibited an identical adrenal-stimulating potency, when assayed for the *in vivo* steroidogenic activity, as shown in Table 3.[25]

Figures 6 and 7 exhibit the electrophoretic patterns of the synthetic human hormone on polyacrylamide gel at pH 4.0 and pH 9.4, respectively, in which those of the porcine hormone preparations are also shown for comparison. There is no significant difference between the two mammalian hormones. Our synthetic preparations of $\alpha_h$-ACTH obtained above were treated with 0.1 M ammonia at 37 °C according to Gráf *et al.*[6] and the product was submitted to polyacrylamide gel electrophoresis at pH 9. A typical result is shown in

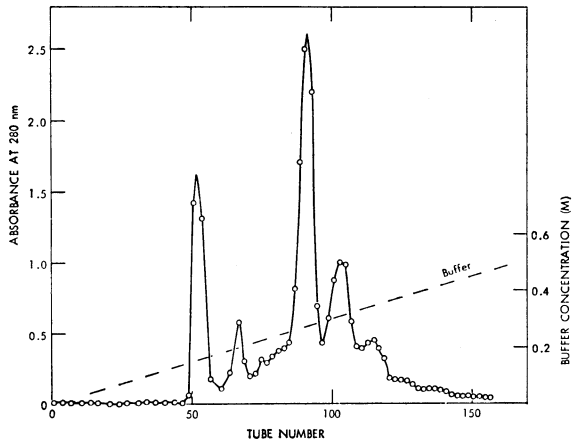



Fig. 10. Carboxymethyl cellulose column chromatography of crude $\alpha_h$-ACTH synthesized *via* route B: sample, 0.34 g; column, CM-52 (Whatman), 2.1×28 cm; buffer, 0—0.6 M ammonium acetate (pH 6.5), 2000 ml; fractionation, 10 ml/tube.

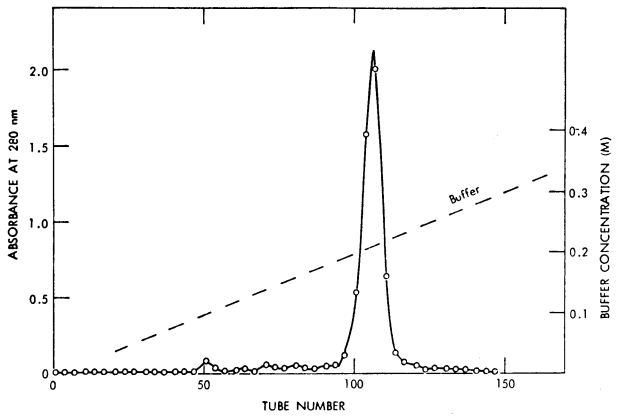



Fig. 11. Rechromatography of partially purified synthetic $\alpha_h$-ACTH on a carboxymethyl cellulose column: sample, 0.13 g, derived from tubes 75—87 in Fig. 10; column, CM-52 (Whatman), 2.1×28 cm; buffer, 0—0.4 M ammonium acetate (pH 6.5), 2000 ml; fractionation, 10 ml/tube.

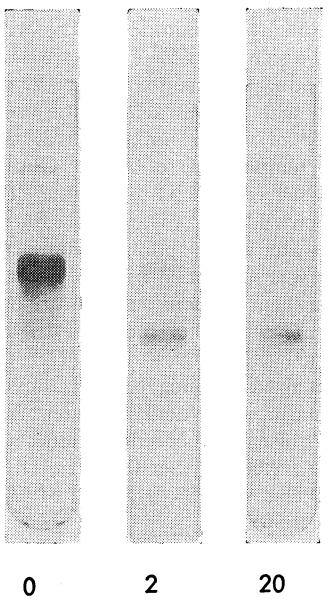



Fig. 12. Electrophoretic patterns of synthetic $\alpha_h$-ACTH after 0, 2, and 20 h of incubation in 0.1 M ammonia at 37 °C: electrophoresis, on 7.5% polyacrylamide gel at pH 9.4; detection, by staining with Amido-Black 10B.

Figure 12, in which the electrophoretic patterns demonstrate that the synthetic hormone is mostly converted into the deamidated form in 2 h and the complete conversion is attained within 20 h. Virtually the same patterns were obtained with our synthetic as well as natural preparations of $\alpha_p$-ACTH as described above. These results clearly indicate that the labile Asn 25 is present intact in our synthetic preparations of both $\alpha_p$-ACTH and $\alpha_h$-ACTH. The optical rotatory dispersion and circular dichroism of synthetic $\alpha_h$-ACTH were very similar to those of the $\alpha_p$-ACTH preparations as shown in Tables 1 and 2.[24] In addition, the *in vivo* steroidogenic potencies of the synthetic $\alpha_h$-ACTH preparations were identical with those of the natural and synthetic preparations of porcine hormone (Table 3). These close similarities observed between the synthetic $\alpha_h$-ACTH and the porcine hormone are consistent with the fact that the primary structure of $\alpha_h$-ACTH is identical with that of $\alpha_p$-ACTH except for Ser 31 (Fig. 1). Although there is no direct evidence to support the identity of our synthetic $\alpha_h$-ACTH with the natural human hormone, the above observations may allow us to conclude that our synthesis of $\alpha_h$-ACTH has been accomplished satisfactorily.

## Experimental

Thin-layer chromatography was performed on "silica gel" plates (Kieselgel $GF_{254}$ or precoated Kieselgel $60F_{254}$, Merck) or "cellulose" plates (precoated Cellulose $G_{254}$, Merck) with the following solvent systems: A chloroform–methanol (85: 15); B, ethyl acetate–acetic acid–water (4: 1: 1); C, 1-butanol–acetic acid–water (4: 1: 2); D, 1-butanol–acetic acid–pyridine–water (30: 6: 20: 24).

*Z–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Ser–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$ (VI).* Compound IVh (Fig. 2; 0.39 g, 0.016 mmol)[15] was dissolved in dimethyl sulfoxide (DMSO)–*N*,*N*-dimethylformamide (DMF) (4: 1, 2.5 ml) and to this was added a solution of Z–Val–Lys(Mhoc)–$N_3$ [derived from the corresponding hydrazide (Fig. 3; 0.22 g, 0.4 mmol)[19c] by the treatment with nitrous acid in the usual manner] in ethyl acetate (10 ml). The mixture was concentrated *in vacuo* at a bath temperature of 5—10 °C to remove ethyl acetate and then stirred at 4 °C overnight. The precipitates which formed upon addition of water were filtered off, washed thoroughly with water and lyophilized from acetic acid. The resulting powder was suspended in ethyl acetate (30 ml) and the suspension was kept at room temperature overnight. The insoluble precipitates were filtered off and dried *in vacuo* to give VI; wt 0.41 g (91%), $[\alpha]_D^{24.5}$ $-16.3\pm1.5°$ (*c* 0.4, DMF). TLC (silica gel): almost homogeneous (ninhydrin, after pretreatment with hydrobromic acid) in system A. Amino acid ratios in acid hydrolysate (theoretical values are presented in parentheses): Asp 2.00 (2), Ser 0.69 (1), Glu 3.89 (4), Pro 2.23 (2), Gly 1.09 (1), Ala 2.98 (3), Val 2.01 (2), Leu 1.00 (1), Tyr 0.61 (1), Phe 1.90 (2).

*Z–Lys(Mhoc)–Pro–Val–Gly–Lys(Mhoc)–Lys(Mhoc)–Arg–(NO$_2$)–Arg(NO$_2$)–Pro–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Ser–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$ (VII).* Compound VI (0.40 g, 0.14 mmol) was hydrogenolyzed palladium in water-saturated 1-butanol for 20 h. The solvent was removed by evaporation *in vacuo* at a bath temperature of 45 °C and the residue was treated with ether to yield the $N^{\alpha}$-free eicosapeptide derivative as an amorphous powder. This was dissolved in DMF (5 ml) along with V (Fig. 3; 0.25 g, 0.14 mmol)[14] and 1-hydroxybenzotriazole (0.080 g, 0.6 mmol), and dicyclohexylcarbodiimide (DCC, 0.12 g, 0.6 mmole) was added. The reaction mixture was stirred at 4 °C for 2 days followed by evaporation *in vacuo*. The residue was treated with ethyl acetate and the insoluble material was filtered off and dried *in vacuo* (0.53 g). The crude product thus obtained was chromatographed on a column of silica gel (30 g, Kieselgel H, Merck) with chloroform–methanol (90: 10) as solvent. Five-g fractions were collected and they were examined by TLC (silica gel, in system A, and sulfuric acid for detection). Tubes 21—50 were pooled and rechromatographed in almost the same manner as above. The fractions containing the desired product as a single component were combined and evaporated *in vacuo* to give a residue which was solidified by trituration with ether; wt 0.30 g (47%), mp 200—205 °C decomp., $[\alpha]_D^{25}$ $-50.5\pm1.8°$ (*c* 0.5, acetic acid).

*H–Lys(Mhoc)–Pro–Val–Gly–Lys(Mhoc)–Lys(Mhoc)–Arg–(NO$_2$)–Arg(NO$_2$)–Pro–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Ser–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$ (VIII).* Compound VII (0.30 g) was hydrogenolyzed over palladium in acetic acid (10 ml) for 24 h. The catalyst was filtered off and the filtrate was lyophilized to give the product which was almost homogeneous in TLC (silica gel, in system B, and ninhydrin as reagent for detection). The product was unreactive to the Sakaguchi reagent wt. 0.29 g, $\lambda_{max}^{MeOH}$ 270 nm ($E_{1cm}^{1\%}$ 62.3). Amino acid ratios in acid hydrolysate: Lys 4.50 (4),[28] Arg 1.67 (2),[28] Asp 1.99 (2), Ser 0.91 (1), Glu 3.94 (4), Pro 4.23 (4), Gly 2.06 (2), Ala 3.02 (3), Val 3.00 (3), Leu 0.94 (1), Tyr 0.74 (1), Phe 1.93 (2).

*Z–Lys(Mhoc)–Pro–Val–Gly–Lys(Mhoc)–Lys(Mhoc)–Arg–Arg–Pro–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Leu–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$) (IXp).* Compound III (Fig. 2; 0.33 g, 0.15 mmol)[14] was dissolved in a mixture of DMSO–DMF (1: 3, 4 ml). The solution was chilled to −20——30 °C and 3.66 M hydrogen chloride in dioxane (0.41 ml) was added. To this was then added isopentyl nitrite (0.022 ml, 0.165 mmol) and the mixture was stirred at −20——30 °C for 15 min. The resulting azide solution was neutralized with triethylamine (0.23 ml, 1.65 mmol) at −40——50 °C and combined with a solution of IVp (Fig. 2; 0.24 g, 0.1 mmol)[15] in DMSO–DMF (1: 4, 2.5 ml). The reaction mixture was stirred at 4 °C for 2 days and then evaporated *in vacuo* at a bath temperature of 45 °C to give a residue which was triturated with water. The crude product (0.54 g) was submitted to partition chromatography on a column (2.8×75 cm) of Sephadex LH-20 with 1-butanol–acetic acid–water (4: 1: 2) as solvent. Three-ml fractions were collected and those containing the desired product as a single component (tubes 46—56), as examined by TLC (silica gel, in system C), were combined, evaporated *in vacuo* at a bath temperature of 45 °C and lyophilized from acetic acid (0.21 g). Tubes 57—61 were combined and rechromatographed in the same manner as above to give an additional quantity of the pure peptide (0.08 g). Thus, the total yield of IXp amounted to 0.29 g (64%); $[\alpha]_D^{23.5}$ $-49.6\pm1.0°$ (*c* 0.9, acetic acid). Amino acid ratios in acid hydrolysate: Lys 3.94 (4), $NH_3$ 1.50 (1), Arg 2.00 (2), Asp 1.90 (2), Glu 4.07 (4), Pro 4.01 (4), Gly 1.94 (2), Ala 2.96 (3), Val 2.89 (3), Leu 2.00 (2), Tyr 0.87 (1), Phe 1.96 (2).

*Z–Lys(Mhoc)–Pro–Val–Gly–Lys(Mhoc)–Lys(Mhoc)–Arg–Arg–Pro–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Ser–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$ (IXh).* Compound III (Fig.

2; 0.33 g, 0.15 mmol)[14] was converted into the corresponding azide by the treatment with isopentyl nitrite in an acid solution in the same manner as described above. The azide solution [in DMSO–DMF (1: 3, 4 ml)] was neutralized with triethylamine (0.23 ml, 1.65 mmol) at −40——50 °C and combined with a solution of IVh (Fig. 2; 0.23 g, 0.1 mmol)[15] in DMSO–DMF (1: 3, 4 ml). The reaction was allowed to proceed at 4 °C for 2 days. The crude product (0.53 g) was purified by partition chromatography on a column of Sephadex LH-20 with 1-butanol–acetic acid–water (4: 1: 2) as solvent, in the manner described above for IXp, to give IXh; wt 0.28 g (63%), $[\alpha]_D^{23.5}$ −47.3±1.0° (*c* 0.8, acetic acid). Amino acid ratios in acid hydrolysate: Lys 3.98 (4), $NH_3$ 1.48 (1), Arg 2.00 (2), Asp 1.99 (2), Ser 0.86 (1), Glu 4.03 (4), Pro 4.11 (4), Gly 2.04 (2), Ala 3.06 (3), Val 3.09 (3), Leu 1.00 (1), Tyr 0.91 (1), Phe 1.92 (2).

*H–Lys(Mhoc)–Pro–Val–Gly–Lys(Mhoc)–Lys(Mhoc)–Arg–Arg–Pro–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Leu–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$ (Xp).* Compound IXp (0.53 g) was hydrogenolyzed over palladium in acetic acid for 2 days. The catalyst was filtered off and the filtrate was lyophilized. The product was chromatographed on a column of silica gel [50 g, Kieselgel 60 (100—230 mesh), Merck] with ethyl acetate–acetic acid–water (4: 1: 1) as solvent. Three-ml fractions were collected and examined by TLC (silica gel, in system C). The fractions containing the desired product as a single component (tubes 33—40) were combined, evaporated *in vacuo* and lyophilized from acetic acid (0.20 g). Tubes 13—32, containing the starting material which had remained intact, were combined, evaporated *in vacuo* and hydrogenolyzed for 24 h. This was combined with tubes 41—47 and chromatographed in the same manner as described above to give an additional quantity of Xp from tubes 44—60 (0.14 g). The total yield amounted to 0.34 g (65%); $[\alpha]_D^{21}$ −46.8±1.4° (*c* 0.6, acetic acid). Amino acid ratios in acid hydrolysate: Lys 4.00 (4), $NH_3$ 1.38 (1), Arg 2.08 (2), Asp 2.00 (2), Glu 4.24 (4), Pro 3.99 (4), Gly 2.08 (2), Ala 3.07 (3), Val 2.94 (3), Leu 2.00 (2), Tyr 0.96 (1), Phe 2.00 (2).

*H–Lys(Mhoc)–Pro–Val–Gly–Lys(Mhoc)–Lys(Mhoc)–Arg–Arg–Pro–Val–Lys(Mhoc)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu$^t$)–Asp(OBu$^t$)–Glu(OBu$^t$)–Ser–Ala–Glu(OBu$^t$)–Ala–Phe–Pro–Leu–Glu(OBu$^t$)–Phe–OBu$^t$ (Xh).* Compound IXh (0.45 g) was hydrogenolyzed over palladium in acetic acid for 2 days. The product was chromatographed on a silica gel column [70 g, Kieselgel 60 (100—230 mesh), Merck] in exactly the same manner as described above for Xp. The desired product was obtained from tubes 56—77 (0.12 g). Tubes 11—55 combined were evaporated and the residue was hydrogenolyzed again. This was rechromatographed as above to give an additional quantity of Xh (0.24 g). The total yield amounted to 0.36 g (82%); $[\alpha]_D^{25.5}$ −41.5±1.6° (*c* 0.5, acetic acid). Amino acid ratios in acid hydrolysate: Lys 3.89 (4), $NH_3$ 1.17 (1), Arg 1.97 (2), Asp 2.09 (2), Ser 0.98 (1), Glu 4.28 (4), Pro 4.36 (4), Gly 2.13 (2), Ala 3.13 (3), Val 3.03 (3), Leu 1.00 (1), Tyr 0.96 (1), Phe 2.06 (2).

*H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–Asn–Gly–Ala–Glu–Asp–Glu–Leu–Ala–Glu–Ala–Phe–Pro–Leu–Glu–Phe–OH, $\alpha_p$-ACTH (Ip).* A solution of II (Fig. 2; 0.15 g, 0.09 mmol) in DMF (2 ml) was chilled in an ice-bath and 1M hydrochloric acid (0.2 ml) was added. This was introduced into an ice-cold mixture of ethyl acetate–ether (1: 1, 50 ml) and the resulting precipitates were collected by filtration, washed with ether and dried *in vacuo*. The decapeptide hydrochloride thus obtained was dissolved in DMF (3 ml) together with *N*-hydroxysuccinimide (0.041 g, 0.36 mmol) and DCC (0.080 g, 0.39 mmol) was added. The mixture was stirred at 4 °C for 20 h, after which it was introduced into ethyl acetate–ether (1: 1, 70 ml). The precipitates which formed were filtered off, washed with ethyl acetate and ether, and dried *in vacuo* to give the decapeptide active ester hydrochloride.

Compound Xp (0.30 g, 0.069 mmol) was dissolved in DMF (5 ml) and to this was added the active ester obtained above. The mixture was stirred at room temperature for 20 h. Removal of the solvent by evaporation *in vacuo* followed by treatment with ether yielded a crude preparation of the protected nonatriacontapeptide (0.46 g). The crude product obtained above was then treated with trifluoroacetic acid (5 ml) at room temperature for 90 min in the presence of anisole (0.46 ml) and 2-mercaptoethanol (0.46 ml). The precipitates which formed upon addition of ether were filtered off, washed with ether and dried *in vacuo*. The crude deblocked peptide thus obtained was dissolved in water and the solution was passed through a column (1.5×11 cm) of Amberlite CG-400 (acetate). The column was washed with additional portions of water. The aqueous solutions combined were lyophilized to give a crude preparation of Ip (0.46 g).

The crude peptide was submitted to chromatography on a column of carboxymethyl cellulose with an ammonium acetate buffer (Fig. 4). The fractions corresponding to a major peak (tubes 98—117) were combined, evaporated *in vacuo* at a bath temperature of 45 °C and lyophilized to a constant weight (0.12 g). Tubes 81—97 and 118—140 were combined and rechromatographed in the same manner as above. The fractions corresponding to the peak of desired peptide were combined, evaporated and lyophilized (0.03 g). These preparations were combined (0.15 g) and chromatographed again on a carboxymethyl cellulose column (Fig. 5). The fractions corresponding to a major peak (tubes 119—130) were combined, evaporated and lyophilized to give a pure preparation of Ip; wt 0.090 g (26%), $[\alpha]_D^{25}$ −88.0±2.4° (*c* 0.5, 2% acetic acid). Lit, $[\alpha]_D^{27}$ −80.2° (*c* 0.3, 1% acetic acid).[13] $\lambda_{max}^{0.1M\ HCl}$ 276 nm ($E_{1cm}^{1\%}$ 16.0), $\lambda_{shoulder}^{0.1M\ HCl}$ 289 nm ($E_{1cm}^{1\%}$10.3); $\lambda_{max}^{0.1M\ NaOH}$ 282.5 nm ($E_{1cm}^{1\%}$ 17.7), 289 nm ($E_{1cm}^{1\%}$ 18.2). TLC (cellulose): homogeneous to ninhydrin and the Ehrlich reagent in system D. Amino acid ratios in acid hydrolysate: Lys 3.73 (4), His 1.09 (1), Arg 2.79 (3), Asp 1.97 (2), Ser 1.91 (2), Glu 5.26 (5), Pro 4.53 (4), Gly 3.11 (3), Ala 3.00 (3), Val 3.02 (3), Met 1.04 (1), Leu 2.00 (2), Tyr 2.08 (2), Phe 3.07 (3). The Tyr/Trp ratio in intact Ip was 2.06, as determined spectrophotometrically.[20]

*H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–Asn–Gly–Ala–Glu–Asp–Glu–Ser–Ala–Glu–Ala–Phe–Pro–Leu–Glu–Phe–OH, $\alpha_h$-ACTH (Ih).* *a) From Compound Xh*: In the same manner as described above for $\alpha_p$-ACTH (Ip), the *N*-hydroxysuccinimide ester hydrochloride, derived from II (Fig. 2; 0.16 g, 0.1 mmol), was coupled with Xh (0.33 g, 0.075 mmol) in DMF (5 ml) at room temperature for 20 h to give the protected nonatriacontapeptide (0.46 g). This was submitted to deprotection with trifluoroacetic acid (5 ml) at room temperature for 90 min in the presence of anisole (0.46 ml) and and 2-mercaptoethanol (0.46 ml) and the product was treated with Amberlite CG-400 (acetate) in water to give a crude preparation of Ih (0.45 g).

The crude peptide was chromatographed on a carboxymethyl cellulose column using an ammonium acetate buffer (Fig. 8). The fractions corresponding to a major peak (tubes 93—111) were combined, evaporated *in vacuo* and lyophilized (0.11 g). Tubes 81—92 and 112—140 were combined and rechromatographed in the same manner as above to afford an additional quantity of the desired peptide

(0.02 g). These preparations combined (0.13 g) were chromatographed again on a carboxymethyl cellulose column (Fig. 9) and the fractions corresponding to a major peak (tubes 119—130) were combined, evaporated and lyophilized to give a pure preparation of Ih; wt 0.080 g (21.5%), $[\alpha]_D^{25}$ $-87.2\pm2.8°$ (*c* 0.5, 2% acetic acid). Lit, $[\alpha]_D^{24}$ $-87.6°$ (*c* 0.26, 2% acetic acid).[11] $\lambda_{max}^{0.1M\ HCl}$ 276 nm ($E_{1cm}^{1\%}$ 16.2), $\lambda_{shoulder}^{0.1M\ HCl}$ 289 nm ($E_{1cm}^{1\%}$ 10.6); $\lambda_{max}^{0.1M\ NaOH}$ 282.5 nm ($E_{1cm}^{1\%}$ 17.3), 289 nm ($E_{1cm}^{1\%}$ 18.0). TLC (cellulose): homogeneous to ninhydrin and the Ehrlich reagent in system D. Amino acid ratios in acid hydrolysate: Lys 4.02 (4), His 1.00 (1), Arg 3.23 (3), Asp 2.04 (2), Ser 2.84 (3), Glu 5.08 (5), Pro 4.42 (4), Gly 3.14 (3), Ala 3.05 (3), Val 3.13 (3), Met 0.98 (1), Leu 1.00 (1), Tyr 2.01 (2), Phe 3.03 (3). The Tyr/Trp ratio in intact Ih was 1.98, as determined spectrophotometrically.[20]

*b) From Compound VIII.* To a solution of VIII (0.31 g, 0.069 mmol) in DMF (5 ml) was added the *N*-hydroxysuccinimide ester hydrochloride, derived from II (0.14 g, 0.089 mmol) in the same manner as described above. The mixture was stirred at room temperature for 20 h and then introduced to ethyl acetate–ether (1: 1, 50 ml) to afford a crude preparation of the protected nonatriacontapeptide (0.43 g). This was then treated with being hydrogen fluoride (20 ml) at 0 °C for 60 min in the presence of anisole (0.5 ml) in the usual manner.[27] Hydrogen fluoride was evaporated *in vacuo* at 0 °C. The resulting residue was dissolved in ice-cold water and the solution was, after being washed with chloroform, passed through a column (1.4×14 cm) of Amberlite CG-400 (acetate). The column was washed with additional portions of water. The aqueous solutions combined were lyophilized to give a crude preparation of Ih (0.34 g).

The crude product thus obtained was chromatographed on a column of carboxymethyl cellulose using an ammonium acetate buffer (Fig. 10). The fractions corresponding to a major peak (tubes 75—87) were combined, concentrated and lyophilized (0.13 g). This was again chromatographed on a carboxymethyl cellulose column in almost the same manner as above (Fig. 11). The fractions corresponding to a single peak (tubes 101—112) were combined and evaporated *in vacuo*. Repeated lyophilization of the residue yielded the desired Ih as colorless fluffy powder: wt 0.100 g (29%), $[\alpha]_D^{24}$ $-86.5\pm4.0°$ (*c* 0.3, 2% acetic acid). $\lambda_{max}^{0.1M\ HCl}$ 276 nm ($E_{1cm}^{1\%}$ 16.0), $\lambda_{shoulder}^{0.1M\ HCl}$ 289 nm ($E_{1cm}^{1\%}$ 10.0); $\lambda_{max}^{0.1M\ NaOH}$ 282.5 nm ($E_{1cm}^{1\%}$ 17.0), 289 nm ($E_{1cm}^{1\%}$ 17.6). TLC (cellulose): homogeneous to ninhydrin and the Ehrlich reagent in system D. Amino acid ratios in acid hydrolysate: Lys 3.75 (4), His 1.14 (1), Arg 2.88 (3), Asp 1.96 (2), Ser 2.51 (3), Glu 4.91 (5), Pro 4.10 (4), Gly 3.12 (3), Ala 2.87 (3), Val 2.97 (3), Met 0.97 (1), Leu 1.00 (1), Tyr 1.99 (2), Phe 2.96 (3). The Tyr/Trp ratio in intact Ih was 1.97, as determined spectrophotometrically.[20]

*Purification of Natural $\alpha_p$-ACTH.* A commercial preparation of $\alpha_p$-ACTH (45 mg)[21] was chromatographed for purification on a column (1.7×10 cm) of carboxymethyl cellulose (CM-52, Whatman) with a 0.1 M sodium acetate buffer (pH 6.2, 1000 ml). The elution was performed by increasing the sodium chloride concentration of buffer linearly from 0 to 0.2 M. Five-ml fractions were collected and their absorption at 280 nm was measured. The fractions corresponding to a major peak (tubes 63—75) were combined and passed through a column (1.1×7 cm) of Amberlite XAD-2, which had been equilibrated with 0.1 M acetic acid, at a flow rate of 35 ml/h. The column was washed with 0.1 M acetic acid (100 ml) and the peptide adsorbed on the column was then eluted with ethanol–0.1M acetic acid (1: 1). Ten ml-fractions were collected and those corresponding to a single peak (tubes 19—33) were combined, evaporated *in vacuo* at a bath temperature of 45 °C and lyophilized; wt 18 mg (recovery 40%),[29] $[\alpha]_D^{21}$ $-84.6\pm3.5°$ (*c* 0.4, 2% acetic acid). $\lambda_{max}^{0.1M\ HCl}$ 276 nm ($E_{1cm}^{1\%}$ 15.5), $\lambda_{shoulder}^{0.1M\ HCl}$ 289 nm ($E_{1cm}^{1\%}$ 10.3); $\lambda_{max}^{0.1M\ NaOH}$ 282.5 nm ($E_{1cm}^{1\%}$ 16.8), 289 nm ($E_{1cm}^{1\%}$ 17.2). TLC (cellulose): homogeneous to ninhydrin and the Ehrlich reagent in system D. Amino acid ratios in acid hydrolysate: Lys 3.84 (4), His 1.05 (1), Arg 2.90 (3), Asp 2.05 (2), Ser 1.79 (2), Glu 4.96 (5), Pro 3.82 (4), Gly 3.11 (3), Ala 3.04 (3), Val 3.00 (3), Met 1.02 (1), Leu 2.06 (2), Tyr 1.98 (2), Phe 3.00 (3). The Tyr/Trp ratio in intact hormone was 1.94 as determined spectrophotometrically.[20]

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28) Upon acid hydrolysis nitroarginine will decompose to give arginine and ornithine. The latter amino acid overlaps the lysine peak under the conditions used.

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